"The Role of Glycosylation in Allergen Recognition"

by

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ABSTRACT

This project is an attempt to have a better understanding of the role of carbohydrates in recognition and uptake of allergens by the innate immune system. Glycosylation analysis of different allergens like Der p 1, Fel d 1, Ara h 1, Ber e 1, Der p 2, Bla g 2, Can f 1, Bromelain and Papain was made using labelled lectins that can detect specific carbohydrate moieties on proteins to reveal their pattern of glycosylation. These experiments showed that all major allergens are glycosylated and this represented a major first step towards demonstrating a link between glycosylation and allergen recognition by the innate immune system. N- and O-glycosylation patterns were predicted in different allergens and a difference in mannosylation and fucosylation was detected between allergens and non-allergenic proteins. We found that the main dominant sugars on allergens are 1,2 1,3 and 1,6 mannose, as detected by GNA lectin. We have also showed that Der p 1 and Der p 2 possess 1,3 fucose in their natural forms, thus concluding that Der p1 and Der p 2 have part of the CCDs which are epitope structures for IgE. O-glycosylation in allergens was also studied giving a better understanding of the whole glycan structure in allergens.

The role of mannosylation in allergen recognition by the immune system was investigated further. Different methods, including recombinant expression, enzymatic and chemical deglycosylation, were optimised to produce glycoforms of Der p 1. A recombinant preparation of Der p 1 produced in *Pichia pastoris* was used as a hypermannosylated form of the allergen. These glycoforms served as useful tools in addressing the nature of glycoallergen recognition by looking

at the uptake of hyper- and hypo-glycosylated preparations by DCs, with confocal microscopy, ELISA and FACS as readouts. Results indicate that deglycosylated forms of Der p 1 exhibited minimal uptake by DCs compared to the natural and hyperglycosylated recombinant allergen. Comparative analysis of the hypermannosylated preparation of Der p 1 and its natural counterpart, possessing less mannan, showed that the recombinant form was taken up more readily by DCs at 37°C and at 4°C. We also showed that these glycoforms bind to the MR subfragment CTLD 4-7-FC, the C-type lectin carbohydrate recognition domain. This binding significantly decreased when the Der p 1 allergen was deglycosylated.

These results were confirmed further using confocal microscopy imaging which also showed that recombinant Der p 1 uptake is immediate, starting at 5 mins of incubation and that a higher quantity accumulates inside the DC compared to natural allergen. Recombinant and natural Der p 1 both co-localised with MR, DC-SIGN and LAMP-2 lysosomal marker, suggesting a key role for these receptors in allergen uptake and a common fate for these preparations inside the DC.

Further experiments were done to show the effect of Der p 1 and Der p 1 glycoforms on TSLP secretion by epithelial cells, which is known to induce Th2 driven immune responses. The results show that TSLP secretion decreases significantly when epithelial cells are challenged with deglycosylated preparations of the same allergen. This may indicate a change in the outcome of adaptive immune responses when a deglycosylated allergen challenges epithelial cells.

In conclusion, this work has demonstrated a link between allergenicity and glycosylation patterns in allergens. It

therefore appears that mannosylation is the dominant sugar moiety associated with allergen uptake and recognition by humans DCs, and this is in line with MR being the main receptor involved in allergen binding by these cells.

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ABBREVIATIONS

Ab: antibody

AP: alkaline phosphatase

APC: allophycocyanin

APCs: antigen presenting cells

Ara h 1: major allergen from peanut (*Arachis hypogaea*)

B cell: B lymphocyte

Ber e 1: Brazil nut allergen (*Bertholletia excelsa)*

Bet v 1: major birch pollen allergen

Bla g 2: major allergen from German cockroach (*Blattella germanic*a)

BM-DCs: bone marrow-derived dendritic cells

BSA: bovine serum albumin

Can f 1: major allergen from domestic dog (*Canis familiaris*)

CCDs: Cross-reactive Carbohydrate Determinants

cDNA: complementary DNA

CLRs: c-type lectin receptors

CR: cysteine rich

CRD: carbohydrate recognition domain

CT: control

CTLD: c-type lectin (carbohydrate) like domain

Cy3: cyanin 3

Cy5: cyanin 5

DCs: dendritic cells

DC-SIGN: denditic cell specific ICAM-3 grabbing non-integrin

D Der p 1: Deglycosylated mutant N52Q of recombinant Der p 1

Deg: deglycosylated

Der p 1: major group1 allergen from *Dermatophagoides pteronyssinus*

Der p 2: major group2 allergen from *Dermatophagoides pteronyssinus*

DFel d 1: Deglycosylated Fel d 1

DSA: *Datura stramonium* agglutinin

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic cid

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescent activated cell sorting

FBS: fetal bovine serum

FcεRI: high affinity receptor for IgE

FcεRII: low affinity receptor for IgE

Fel d 1: major allergen from domestic cat (*Felis domisticus*)

FITC: fluorescein isothiocyanate

FNII: fibronectin type 2

GlcNAc: N-Acetylglucosamine

GNA: *Galanthus nivalis* agglutinin

HDM: house dust mite

HLA: human leukocyte antigen

HRP: horseradish peroxidase

IFN: interferon

ICAM: intracellular adhesion molecule

Ig: immunoglobulin

IgE: immunoglobulin E

IL: interleukin

kDa: kilo Dalton

LAMP-2: Lysosomal-associated membrane protein 2

LeX: lewis x

mAb: monoclonal antibody

MACS: magnetic activated cell sorting

mDCs: myeloid dendritic cells

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

MMA: *Maackia amurensis* agglutinin

Mo-DCs: monocyte-derived dendritic cells

MR: mannose receptor

MW: molecular weight

NK: natural killer cells

nDer p 1: natural Der p 1

nFel d 1: natural Fel d 1

OD: optical density

PAA: polyacrylamide

PAMPs: pathogen associated molecular patterns

P. Pastoris: *Pichia pastoris*

PBMCs: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline-tween

PBA: phosphate buffered albumin

PC5: phycoerythrin cyanin 5

PCR: polymerase chain reaction

PE: phycoerythrin

PNA: Peanut agglutinin

PRR: pattern recognition receptor

rDer p 1: recombinant Der p 1

SDS-PAGE: sodium dodecylsulphate-polyacrylamide gel electrophoresis

SNA: *Sambucus nigra* agglutinin

T cell: T lymphocyte

Th cell: T helper lymphocytes

TCR: T cell receptor

TNF: tumor necrosis factor

TLR: Toll Like Receptors

Treg: regulatory T cell

w/v: weight per volume

v/v: volume per volume

WT: wild type

Publications arising from this thesis

1. Royer, P. J., Emara, M., Yang, C., Al-Ghouleh, A., Tighe, P., Jones, N., Sewell, H. F., Shakib, F., Martinez-Pomares, L. & Ghaemmaghami, A. M. (2010) The mannose receptor mediates the uptake of diverse native allergens by dendritic cells and determines allergen-induced T cell polarization through modulation of IDO activity. J Immunol, 185, 1522-31.

2. The role of Der p 1 glycosylation in its uptake by dendritic cells. (**Manuscript in preparation**)

Conferences attended and awards:

1. This work has won the Abstract session for the 29th Congress of the European Academy of Allergy and Clinical Immunology entitled 'Hot Topics in Allergy' (EAACI), held in London, 5-9 June 2010.

2. Poster session at the Young Scientist Symposium (YLS) on the 26th of May 2010 in London. ''Role of glycosylation in allergen recognition''.

3. This project represented the school of Molecular and Medical Sciences in a poster on the 40th anniversary of the opening of Nottingham Medical School. 15th Oct 2010, Nottingham, ''The role of glycosylation in allergen recognition''.

CHAPTER ONE: Introduction

1.1 The immune system:

The environment that we live in is heavily populated with pathogens and allergic substances that constantly challenge our immune system, which has a primary aim of protecting the host against infectious microbes. The immune response therefore uses a complex array of molecules to control and eliminate these pathogens. The human defence system consists of a first line of physiological barriers like the skin and lysozymes of tears and saliva. The second line of defence is the innate immune system which boosts the protection provided by the first line by recognising conserved microbial components that are shared by large groups of pathogens, and this is facilitated through different kinds of cells like macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) cells, and NK T cells (Fig 1-1). The third line of defence is adaptive immunity involving T and B lymphocytes. Any failure of these mechanisms can lead to infection (Chaplin, 2006, Roitt and Delves, 2001, Turvey and Broide, 2010).

As the immune system uses potent effecter mechanisms to clear pathogens, it is crucial to avoid destroying its own tissues. This is referred to as self tolerance which leads to the avoidance of reactions against self antigens and is expressed in many parts of the innate and adaptive immune responses. Failure of self tolerance would lead to autoimmune diseases (Kay, 2000, Chaplin, 2010).

Upon encountering a pathogen the first line of defence is the epithelial cells which express tight cell junctions and cadherin-mediated cell interactions. The secreted mucus layer and the epithelial cilia all help to clear the pathogen (David, 2010, Hammad and Lambrecht, 2008, Chaplin, 2010). The innate immune defence works with the physical barrier to fight the foreign antigen through complement proteins, cytokines that regulate the function of other cells and chemokines that attract inflammatory leukocytes. The innate immune system includes membrane-bound receptors that recognise pathogen associated molecular patterns (PAMPs) and cytoplasmic proteins that bind these molecular patterns expressed on the surfaces of invading microbes (Turvey and Broide, 2010). For example, mannose binding lectin binds mannose containing carbohydrates on microbes, thus triggering the activation of the complement

cascade to clear pathogen. Another example is Toll Like Receptors (TLRs) which recognise diverse microbial epitopes on pathogens and enable the innate immune system to distinguish different groups of pathogens and to induce an appropriate cascade of adaptive responses (Turvey and Broide, 2010, Hammad and Lambrecht, 2008).

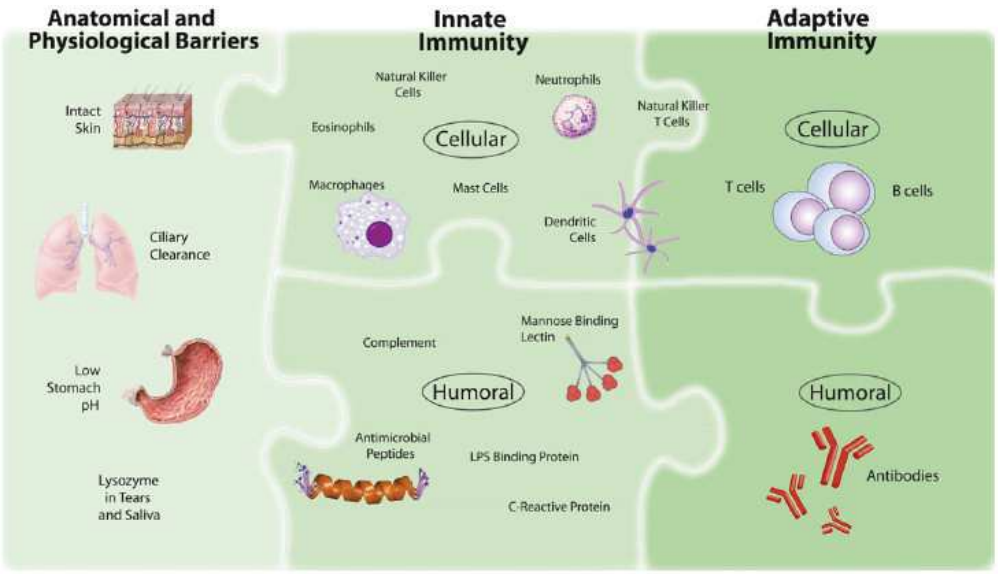


Fig 1-1: The human defence system consists of three lines of defence; each uses a complex array of elements in trying to clear infection. (Turvey and Broide, 2010)

The cellular elements of the innate immune system includes neutrophils, monocytes and macrophages which are phagocytic and can clear the pathogen through binding to immunoglobulin, complement or both. They also produce cytokines like IL-12 and IFN-γ that aid in the regulation of

adaptive immune responses. Some of the newly indentified cytokines in innate immunity include IL-25, IL-33 and TSLP (Thymic Stromal Lymphopoietin) which induce the secretion of Th2 inflammatory cytokines (Fig 1-2) (Kim et al., 2010). NK cells are a distinct lineage of lymphoid cells that kill virally infected cells. Also, dendritic cells (DCs) play a vital role in between the innate and adaptive immune system as antigen presenting cells (APCs). They express MHC I and MHC II, which facilitate recognition of processed antigen by T cells (Fig 1-2) (Kim et al., 2010, Chaplin, 2010).

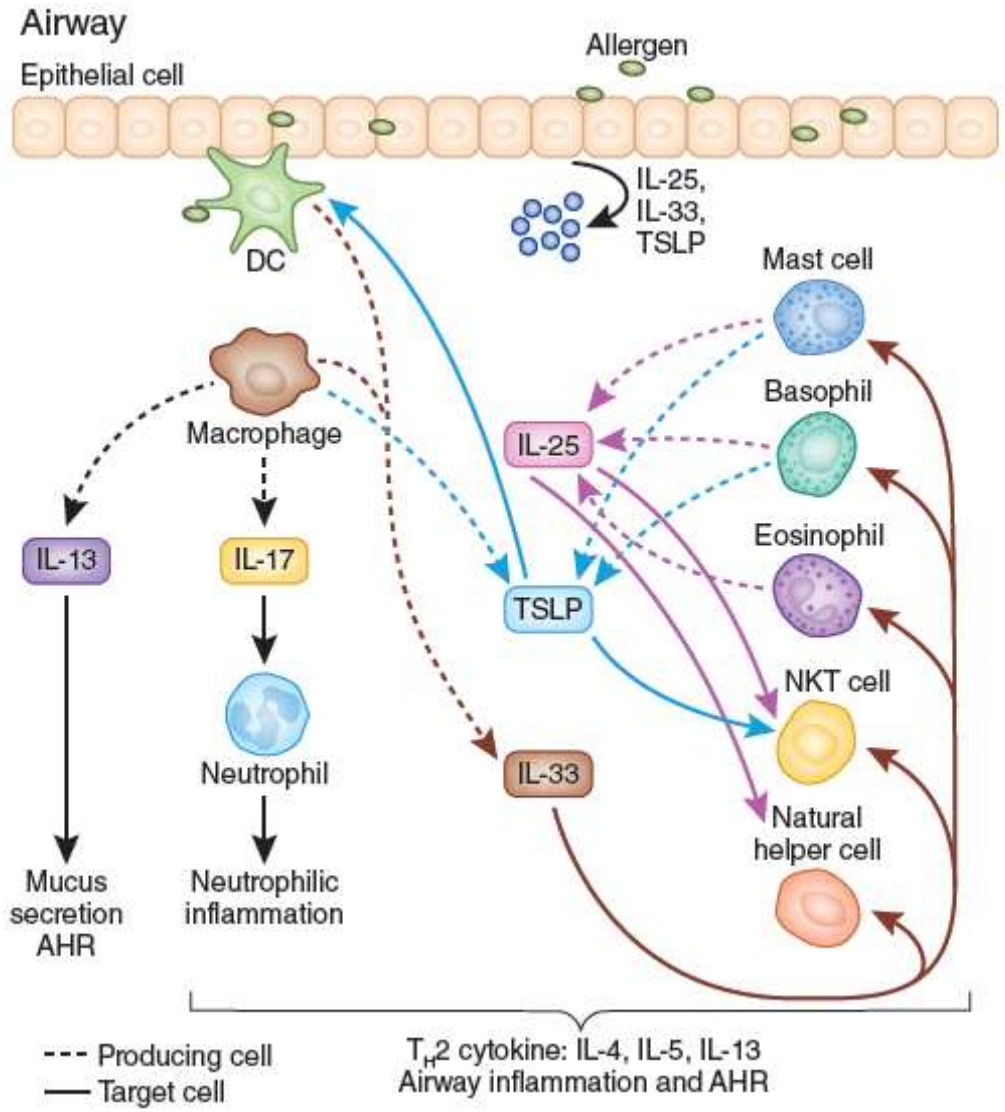


Fig 1-2: The cellular elements of the innate immune defence system. (Kim et al., 2010)

The adaptive immune system includes T and B lymphocytes with their antigen specific receptors. T cells use these receptors to identify cells infected by pathogens and destroy them. T cells recognise molecular complexes of the

pathogenic antigen and a self-structure. The self-structures are either MHC I molecules that bind fragments of proteins that have been synthesised within the cell or MHC II molecules that bind to fragments of proteins that have been ingested by the cell and proteolytically processed. Subsequently, T cells differentiate to CD8 cytotoxic T cells that kill the infected cell or DC4 T helper cells that regulate the cellular and humoral immune responses (Roitt and Delves, 2001, Chaplin, 2010). CD4 T cells can differentiate into Th1, Th2 or Th17 depending on the nature of the cytokines present. IL-12 and IFN-γ produced by macrophages or NK cells induce differentiation towards Th1; IL-4, IL-5, IL-9 and IL-13 induce differentiation towards a Th2 immune response; a connection between high levels of thymic stromal lymphopoietin (TSLP) secretion and the induction of Th2 immune responses has been established. TSLP is an IL-7-like cytokine which is released by primary human epithelial cells in response to certain microbial products. TSLP can activate DCs and increases their expression of the ligand for the T cell costimulatory molecule OX40 thus enhancing Th2 immune responses. Other cytokines have been shown to enhance Th17

inflammatory responses like TGF-β and IL-6 (Asarch et al., 2008, Chaplin, 2010, Bettelli et al., 2007, Kim et al., 2010). B cells on the other hand function to produce antigen specific immunoglobulins (Igs) that attack the foreign antigens and facilitate their presentation to T cells. B cell maturation and Ig production are dependent on Th cell cytokine production. T cell-derived IL-10 causes switching to IgG1 and IgG3. IL-4 and IL-13 cytokines cause switching to IgE, while TGF-β causes switching to IgA. IFN-γ or some other undefined product of Th1 cells appears to induce switching to IgG2 (Chaplin, 2010).

1.1.1 Atopy and Allergy:

The term 'allergy' was introduced in 1906 by Von Pirquet in trying to describe 'uncommitted' biological responses in the body leading either to immunity or hypersensitivity reactions. Since then, allergic diseases have been the focus of interest for scientists in trying to unveil the dogma behind 'what makes an allergen'. The answer to this question is important in understanding the mechanism behind hypersensitivity responses and to design a better therapeutic approach to allergic diseases like asthma, atopic

eczema and allergic rhinitis which are common causes for chronic ill-health (Robinson et al., 1997, Kay, 2000). It is thought that allergens might possess common surface features or glycosylation patterns that enable the innate immune defence to recognise them as Th2 inducers. The identification of common structural features among allergens is important in understanding the role of allergens in inducing hypersensitivity reactions (Shreffler et al., 2006). Research on the glycosylation features of allergens have suggested that carbohydrates displayed by allergens could be unique in structure or orientation and thus being recognised by the mannose receptor on dendritic cells, thereby favouring Th2 polarisation and allergy (Shakib et al., 1998, Shreffler et al., 2006, Altmann, 2007, Taylor et al., 2005). In general, non-atopic individuals will exhibit low level of immunological response to allergens, while atopic individuals will mount an exaggerated immune response to these generally innocuous substances, which has all the hallmarks of type I hypersensitivity, such as production of high levels of IgE antibody and Th2 type cytokines (Kay, 2000).

The immune system is the sole barrier of defence against infection; it prevents the pathogen from breaching the body

by providing immediate innate immunity and a later specific adaptive immunity. Innate immunity involves inflammation, complement system and special leukocytes like mast cells, eosinophils, basophils, natural killers and phagocytes. The adaptive immune cells involve B and T lymphocytes. B cells express different antibodies directly attacking the foreign antigen, while T cells, which are divided into two subtypes, cytotoxic T cells and helper T cells, recognise the foreign antigen by processing and presenting the antigen by a self receptor called the major histocompatibility complex (MHC). Cytotoxic T cells recognise antigen coupled with MHC I, while helper T cells recognise antigen coupled with MHC II. The Th cells themselves differentiate into three subtypes called Th1, Th2 and Th17 according to their cytokine production upon stimulation (Tada T, 1978, Bettelli et al., 2007, Asarch et al., 2008, Bird, 2008). It is thought that IFN-γ and IL-12 stimulate Th1 immune responses which fight intracellular pathogens, while production of IL-4, IL-5, IL-13 and TSLP stimulate Th2 immune response which attack extracellular pathogens by regulating antibody production (Fig 1-3) (Roitt and Delves, 2001, SL., 2007, Reiner., 2007, Minton, 2008, Hammad and Lambrecht, 2008).

Allergens can trigger the production of TSLP by airway epithelial cells, which induces DCs to recruit Th2 cells into the airway (Fig 1-3) (Ziegler and Artis, 2010, Hammad and Lambrecht, 2008, Li et al., 2010). Th 17 cells are thought to be regulated by IL-23, these cells secrete IL-17 and IL-22 which are inflammatory cytokines suggesting a role for these cells in mediating inflammation and in protection against extracellular pathogens. Investigators later found that transforming growth factor (TGF)-β1, IL-6, and IL-15 stimulated initial Th17 differentiation from naive T cells (Asarch et al., 2008, Bettelli et al., 2007).

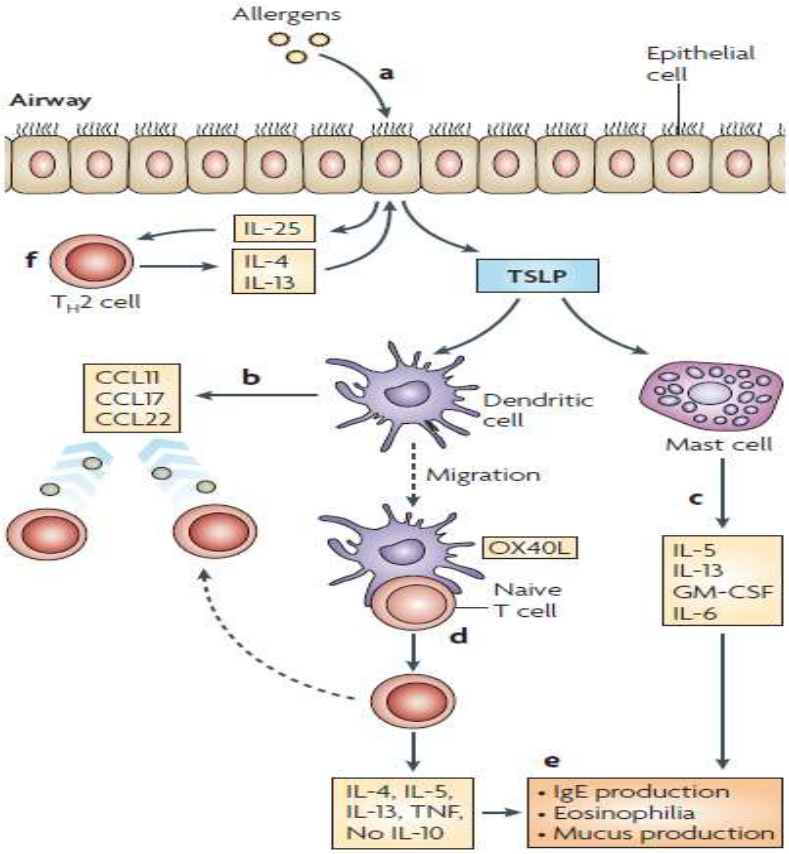


Fig 1-3: The role of TSLP in driving DC maturation towards supporting Th2 immune responses. **A.** Allergen triggering the activation of *Tslp* gene transcription. **B.** TSLP induces immediate innate immune functions in dendritic cells (DCs) leading to chemokine-driven recruitment of Th2 cells. **C.** In mast cells, there is immediate release of the Th2-type effector cytokines that can attract and activate eosinophils in a T-cell independent manner. **D.** Following innate immune induction, TSLP triggers the maturation of DCs so that they migrate to the mediastinal lymph nodes and induce the polarisation of inflammatory Th2 cells in an

OX40L-dependent fashion. **E.** The effector cytokines produced by adaptive Th2 cells and mast cells trigger the salient features of asthma. **F.** Effector cytokines can also perpetuate TSLP-driven inflammation by further triggering the release of TSLP by airway epithelial cells. This process is enhanced by epithelial-cell production of the pro-allergenic cytokine IL-25. CCL11, CC-chemokine. (Hammad and Lambrecht, 2008)

All this protection depends on the ability of the immune system to distinguish between self and non-self antigens. Allergens can destabilise the immune system; they have the ability to induce IgE responses, which involve the sensitisation of B, T and dendritic cells. They can also induce clinical responses on subsequent exposure which involves immediate and late hypersensitivity reactions. When the immune system produces large amounts of IgE, the individual is said to be atopic. The most significant characteristic of atopy is having high IgE levels in patients' serum against a specific allergen (Navarro et al., 2007, Platts-Mills et al., 2007).

Atopy and allergy are often used interchangeably but they are different. Allergy is an excessive immune response to a

foreign antigen regardless of the mechanism, while atopy is a profuse IgE mediated immune response (Johansson et al., 2004, Dreborg, 2003). Atopy in general includes eczema, allergic conjunctivis, allergic rhinitis and asthma (Andrew, 2004). Upon inhaling aero-allergens, non-atopic individuals have low grade of immunological responses, they produce IgG1 and IgG4 antibodies and they also mount a Th1 immune response by the production of IFN-γ and IL-12, while atopic patients mount an exaggerated IgE immune response and tend to polarise a Th2 immune phenotype (Figure 1-1) by producing IL-4, IL-5 and IL-13 (Ebner et al., 1995, Wierenga et al., 1990, Bird, 2008). Factors influencing whether Th1 or Th2 will dominate immune responses are the dose of allergen, length of exposure and the avidity between T cells and antigen presenting cells (Rogers and Croft, 1999). It is suggested that atopy and allergic asthma are less frequent in people exposed to agents in soil, air and water as bacteria and viruses, the so called hygiene hypothesis. This will produce an IL-12 rich environment, thus enhancing Th1, rather than Th2, immune responses (Kapsenberg et al., 2000, Kay, 2000, Platts-Mills et al., 2007, Rogers and Croft, 1999, Minton, 2008).

The term hypersensitivity is used to describe the exaggerated adaptive immune response against a specific antigen causing tissue damage. Hypersensitivity reactions were classified into four classes according to Coombs and Gell. Types I, II and III are antibody mediated while type IV is T cell mediated (Johansson et al., 2004). Type I hypersensitivity underlies all atopic and most allergic disorders as it is caused by a high IgE influx, while in type II IgM and IgG antibodies cause direct cell destruction involving mostly endogenous antigens. Type III hypersensitivity is caused by IgG antibodies that are mainly directed against soluble antigens forming immune complexes, and type IV includes Th1 immune responses and is considered to be T cell mediated (Dreborg, 2003, Johansson et al., 2004, Roitt and Delves, 2001, Brooks et al., 2004).

1.1.2 Type I hypersensitivity:

Type I hypersensitivity, which is also called anaphylactic or immediate hypersensitivity, is characterised by an immediate damaging response to a secondary exposure of an allergen resulting in recruitment of mast cells and

basophils and production of IgE antibody causing extreme inflammatory responses. When mast cells are triggered they release histamine, prostaglandins and leukotrienes. Good examples for this type of hypersensitivity are allergic conjunctivitis, allergic rhinitis and asthma (Saito, 2007, Roitt and Delves, 2001, Gould and Sutton, 2008).

The mechanism of the reaction involves IgE production in response to certain allergens. Upon inhalation of an allergen, Th2 driven responses occur. B cells switch to IgE isotype production upon exposure to the Th2 cytokines IL-4 and IL-13. Basophils and mast cells become sensitised with IgE and upon subsequent exposure to allergens undergo degranulation and release inflammatory mediators like histamine that cause vasodilatation and smooth muscle contraction. This results in an immediate inflammatory response that starts within seconds causing rhinorrhea, itching and anaphylaxis; it is then followed by a late phase response that takes six to eight hours to develop. At a later stage eosinophils, platelets and neutrophils are also triggered. Platelet activation factor (PAF) amplifies the reaction by causing platelet aggregation and more release of histamine. Eosinophil and neutrophil chemotactic factors

attract eosinophils and neutrophils, respectively (Akdis, 2006, Roitt and Delves, 2001, Brooks et al., 2004).

1.1.3 Role of different cell types involved in the allergic cascade:

In some cases allergy is a systemic reaction involving multiple organ systems. It begins when the body encounters an allergen which results in type I hypersensitivity reactions where B cells produce IgE antibodies and T cells regulate IgE production by producing different cytokines like IL-4, IL-5, IL-9 and IL-13. Mast cells and basophils undergo degranulation and release histamine causing vasodilation of capillaries (Kay, 2000, Saito, 2007, Gould and Sutton, 2008).

Other cells involved in allergy are dendritic cells (DCs) which are specialised antigen presenting cells (APCs) that are able to recognise the antigen at the site of entry and present it to naive T helper cells in draining lymph nodes, which will lead to the development of Th1 or Th2 immune responses. It has been shown that monocyte derived dendritic cells induce a Th1 response; whereas $CD4^+$ $CD3^-$

$CD11c^-$ derived dendritic cells induce a Th2 immune response (VINEY, 1999). Others argue that it is the amount of IL-12 secreted by dendritic cells that determine the immune response pathway that follows (Kalinski et al., 1999).

Research on the effect of Der p 1 (a major house dust mite allergen) on dendritic cells showed that enzymatically active Der p 1 cleaves CD40 from the DC surface leading to suppression of IL-12 secretion and this initiates the establishment of a Th2 cytokine profile upon co-culturing these DCs with naïve T cells (Ghaemmaghami et al., 2002). DC use numerous C-type lectins, such as MR, DC-SIGN, dectin-1, langerin, and DEC-205, for antigen uptake. (Erbacher et al., 2009). These lectin receptors are expressed by DCs and are involved in the recognition and capture of many glycosylated self-antigens and pathogens.

The mannose receptor (MR), which is a C-type lectin receptor, recognises a wide range of both endogenous and exogenous ligands and has been implicated in pathogen recognition (Gazi and Martinez-Pomares, 2009). It is composed of 3 regions; an extracellular region containing a cysteine rich domain (CR domain), a domain containing fibronectin type two repeats (FNII) and multiple C-type

lectin-like carbohydrate recognition domains (CTLDs), a transmembrane domain and a short cytoplsmic tail (Fig 1-4) (Taylor et al., 2005). MR recognises mannose, fucose and N-acetylglucosamine on the surfaces of microorganisms and carbohydrate recognition is mediated by CTLD 4-7 (Kerrigan and Brown, 2009, Taylor et al., 2005).

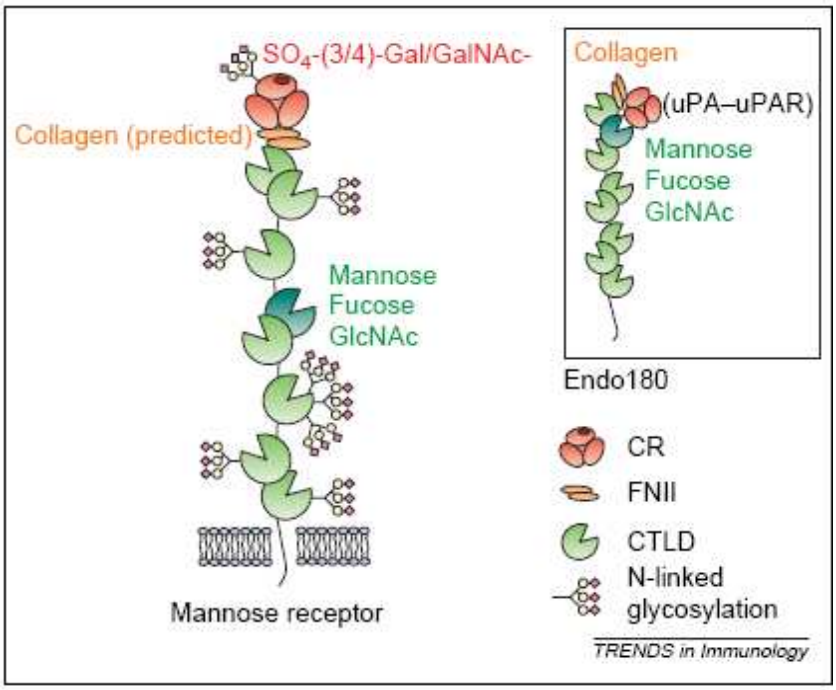


Fig 1-4: Structure of the Mannose Receptor (Taylor et al., 2005).

There is an abundant expression of MR on monocyte derived DCs, which leads to the assumption that MR is used as an antigen recognition tool by these cells. When DCs are treated with PAM-1 monoclonal antibody that is specific to MR, DCs could not produce Th1 recruiting chemokines but

released Th2 and T regulatory cell recruiting chemokines, which are negative regulators of Th1 responses (Fig 1-5). When MR and TLR2 were co-expressed on the same cell, IL-8 secretion was detected, thus MR has been found to be involved in the triggering of a regulatory-promoting phenotype in human DCs (Gazi and Martinez-Pomares, 2009). It has been suggested that MR on DCs could play a role in the recognition and uptake of Der p 1 allergen and this has been shown through labeling Der p 1 and observing its uptake by DCs. The uptake of Der p 1 can be inhibited by mannan, the natural ligand for MR, thus demonstrating the importance of MR in the recognition of Der p 1 (Deslee, Gt et al. 2002). The silencing of MR expression on monocyte-derived DCs reverses the Th2 cell polarisation bias, driven by Der p 1 allergen exposure, through upregulation of IDO activity (Deslee et al., 2002, Royer et al., 2010).

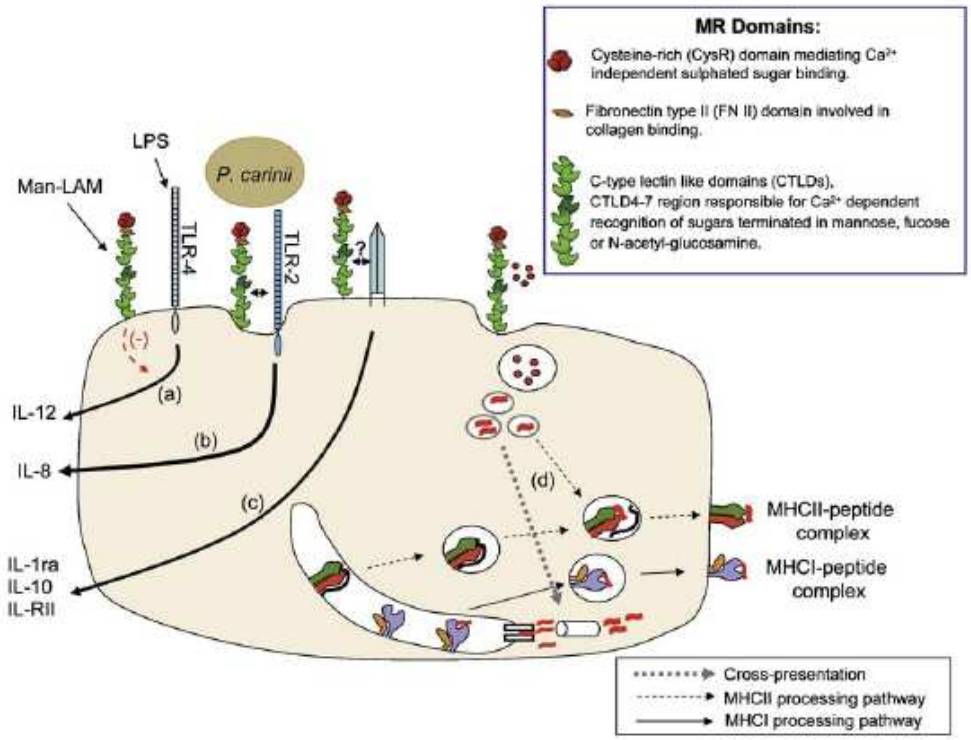


Fig 1-5: MR co-operation with other signalling receptors to control cytokine secretion. MR engagement by Man-LAM has a negative effect on the production of IL-12 in response to LPS in human DC (**a**). Co-expression of MR and TLR2 is required for IL-8 production in response to *P. carinii* (**b**). Additionally, engagement of MR by a specific mAb or selected ligands leads to the production of anti-inflammatory mediators (**c**). MR-mediated internalisation favours cross-presentation (**d**) in addition to MHCII-mediated presentation of exogenous antigens (Gazi and Martinez-Pomares, 2009).

Another C-type lectin receptor on DCs is DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin), which was recently discovered as a mannose specific C-type lectin which was found to bind ICAM-3 (DC specific intercellular adhesion molecule 3) with high affinity, thus initiating the contact of DCs with resting T cells (Geijtenbeek et al., 2000). It was reported that Der p 1 cleaves the extracellular portion of DC-SIGN indicating that it is also a target for Der p 1 allergen and is therefore implicated in driving allergic responses (Furmonaviciene et al., 2007).

1.2 The House Dust Mite Allergens:

There are different kinds of allergens that induce atopic allergic diseases; these include indoor allergens, pollen allergens and food allergens. The most prevalent indoor allergens are the house dust mite allergens of the species *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* which are categorised into group I and group II allergens (Kay, 2000, Thomas W. R. et al., 2002). Der p 1 and Der f 1 are cysteine protease allergens and are said to be within group I allergens, while group II allergens, which include Der p 2 and Der f 2, are still being studied for their functional and structural similarities (Chapman et al., 2007). The cysteine protease activity in house dust mite

allergens has been directly linked to cleaving cell surface molecules that control IgE synthesis, thus promoting Th2 immune responses that lead to asthma (Furmonaviciene et al., 2007, Thomas W. R. et al., 2002, Shakib et al., 1998, Deb et al., 2007).

In recent years many allergens have been produced as recombinant proteins expressed in bacteria and yeast, leading to a growing family of recombinant allergens like Bet v 1, Der p 1, Der p 2, Der f 1 and Ara h 1 (Chapman et al., 2000, Best et al., 2000). These have been shown to be a useful tool in studying T and B cell recognition of allergens as well as IgE regulation. The three dimensional structure for allergens, including Bet v 1, Der p 1, Der p 2 and Ara h 1, has been determined by NMR, X-ray crystallography and computer based molecular modelling. Sequence homology searches have revealed the biological function of different allergens (Nishiyama et al., 1995, Chapman et al., 2000, Chua et al., 1988).

1.2.1 The Der p 1 allergen:

Epidemiological studies showed a relationship between exposure to house dust mite allergens and the increase in asthma cases (Kay, 2000, Richardson et al., 2005, Sharma et al., 2003). Allergens from the house dust mite

Dermatophagoides pteronyssinus are commonly associated with hypersensitivity reactions. This type of allergy affects 30% of the population in developed countries, which is due to the increased exposure to indoor allergens. There are fifteen different Der p allergens that have been identified to date according to the allergen nomenclature committee http://www.allergen.org/Allergen.aspx, and it has been shown that homes of people suffering from house dust mite allergy contain high levels of Der p 1 allergen, which is considered one of the major allergens in western countries (Guy et al., 1995, Adnan et al., 1996, Tsai et al., 2005, O'Neil et al., 2006, Weghofer et al., 2005, Thomas W. R. et al., 2002).

Der p 1 is a 25 kDa allergen with cysteine protease activity. It was identified in 1969 by Voorhorst and is now considered the most significant house dust allergen. It is produced in the mid gut of the mite and found in mite faecal pellets (Voorhorst R 1969). The role of Der p 1 is to help in digestion of food substrates in mites, like human keratin, and it is found in high concentrations in house dust mite faeces, which when inhaled could provoke allergy attacks and asthma.

It has been shown that up to 90% of individuals who are sensitised to house dust mite allergens are actually responsive to Der p 1 (Sharma et al., 2003, Richardson et al., 2005, Thomas and Smith, 1998). Wan *et al,* (2000) found that Der p 1 was able to break the tight junctions of the epithelial cells, suggesting a role for Der p 1 in lung epithelium permeability which contributes to sensitisation and allergic responses. Also, it has been clearly shown that Der p 1 affects both innate and adaptive immune responses. For instance, by using its cysteine protease activity, it induces mast cell and basophil degranulation which influence IgE antibody production. It has also been shown that Der p 1 can cleave cell surface molecules that control IgE synthesis, like CD25 on T cells, CD23 on B cells and CD40 on dendritic cells (Ghaemmaghami et al., 2002, Shakib et al., 1998). Der p 1 was also shown to cleave DC-SIGN on dendritic cells and DC-SIGNR expressed by endothelial cells (Furmonaviciene et al., 2007). Investigators have shown that proteolytically active Der p 1 suppresses IFN-γ and enhances IL-4 production by both CD4 and CD8 cells, thus enhancing IgE production by B cells and supporting an overall type 2 cytokine response (Ghaemmaghami et al., 2001). It was shown that when Der

p 1 cleaves CD40 from dendritic cells, that will lead to the production of less IL-12 and will stimulate CD4 T cells to produce more IL-4 and less IFN-γ which again directs the immune system towards a Th2 immune response (Figure 1-6).

Der p 1, as well as Der f 1, has been shown to act on lung epithelium by cleaving the lung surfactant proteins (SP) A and D which are carbohydrate binding proteins. This results in diminishing their ability to bind and neutralise glycoallergens (Deb et al., 2007). Der p 1 also inhibits α_1-antitrypsin, which is an anti-protease forming part of the mucosal innate defence system. The neutralisation of α_1-antitrypsin could therefore weaken host defences against epithelial cell damage, and this may harm airway tissues (Sharma et al., 2003).

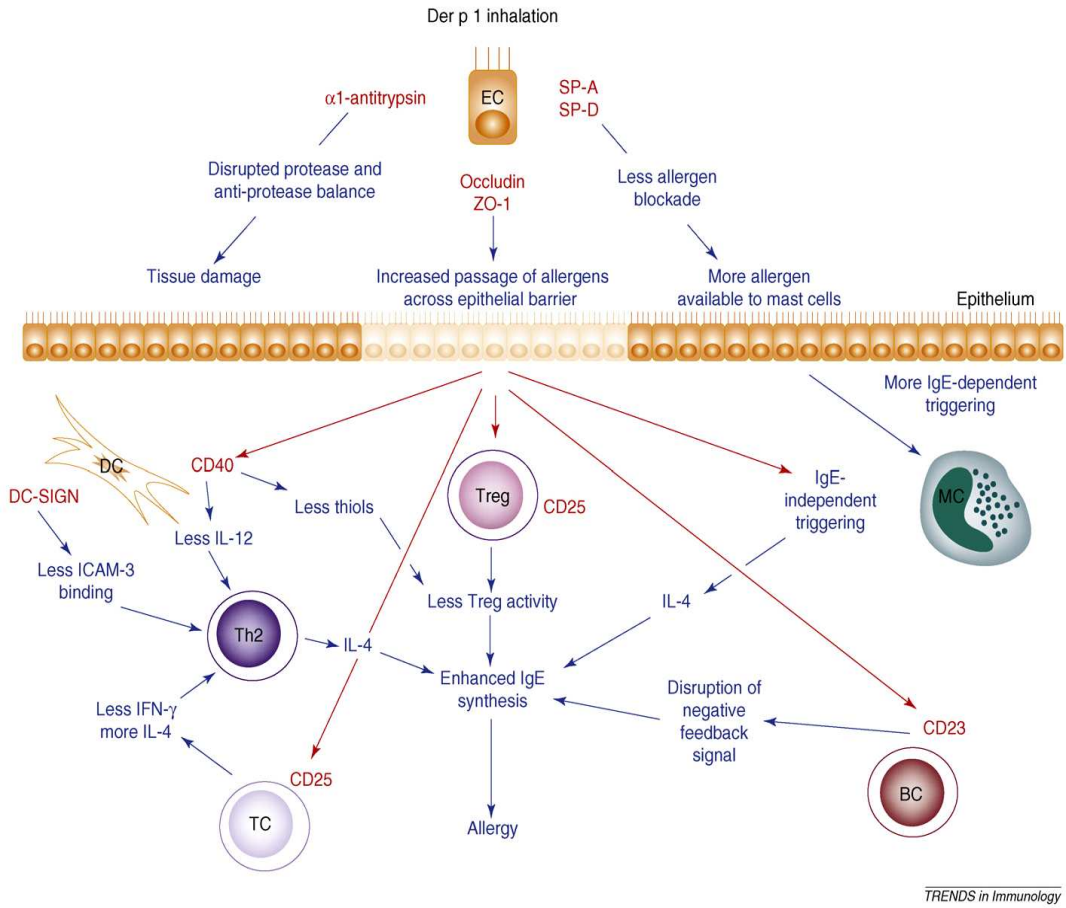


Fig 1-6: The protease activity of Der p 1 favours hyper-IgE production and allergy. Der p 1 proteolytically digests surface molecules on dendritic cells (DC), mast cells (MC), T cells (TC), T regulatory (Treg) cells and B cells (BC) leading to uncontrolled IgE production and allergy (Shakib et al., 2008).

1.2.2 The molecular structure of Der p 1:

The molecular structure of Der p 1 has been the subject of many studies. The gene encoding Der p 1 has been cloned and sequenced, and the cDNA clone coding for Der p 1 was

first sequenced by Chua *et al.,* (1988). It encodes for a 222 residue mature protein having a molecular weight of 25,371 and containing four potential *N*-glycosylation sites. Der p 1 is produced in the mite as an enzymatically inactive proenzyme that becomes active after cleavage of the propeptide. When Der p 1 is extracted from the mite faeces it is in the mature form (nDer p 1) (Chua et al., 1988, Meno et al., 2005, de Halleux et al., 2006). Meno *et al,* (2005) were able to produce a recombinant form of Der p 1 that is hypoglycosylated using an N132E mutation that rendered the protein non-susceptible to glycosylation at this site. The recombinant form was expressed in *Pichia pastoris*. This study provided a detailed description of the crystal structure of the pro-form of Der p 1 and the antibody binding properties of pro and mature Derp 1.

De Halleux *et al* also determined the 3-dimentional crystallographic structure for both glycosylated and unglycosylated forms of recombinant Der p 1 by producing a recombinant pro-Der p 1 exhibiting allergenicity similar to its natural counterpart. They confirmed this by demonstrating that the recombinant form was recognised by human IgE and activated basophils just as well as the natural one. The crystal structure showed that the molecule

preserves a C1 cysteine protease fold consisting of two domains and a catalytic site between them. This study also showed the existence of a magnesium binding site in Der p 1 and determined the epitope binding regions (Fig 1-7). Better understanding of the molecular structure of Der p 1 will clearly offer the chance to design specific inhibitors of allergens both *in vitro* and *in vivo*.

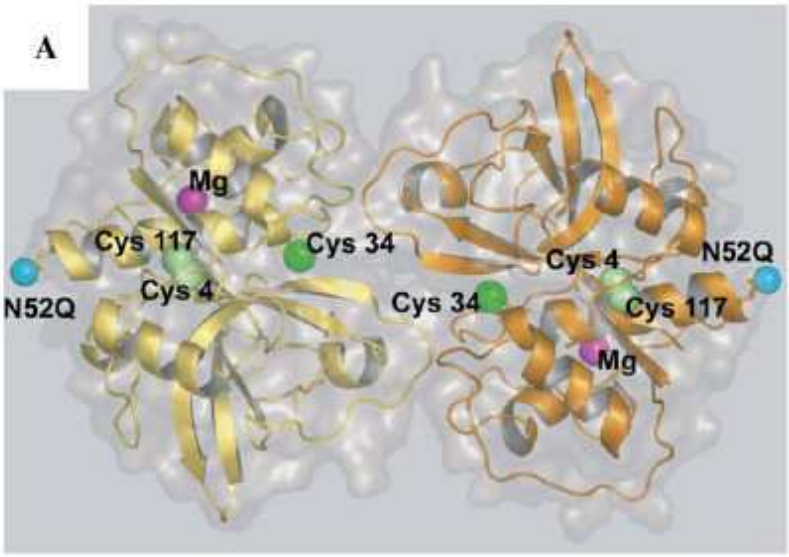


Fig 1-7: Ribbon diagram of mature Der p 1. A view of the Der p 1 dimer (de Halleux et al., 2006).

1.2.3 Recombinant Der p 1:

Almost a decade ago several groups tried isolating and analysing cDNA coding for allergens in order to identify proteins inducing IgE responses in humans. It was then that similarities between allergens and other proteins were

discovered. Der p 1, for example, was considered a papain-like protease. In recent years, a number of different recombinant allergens have been produced which have functional and immunological similarities to their native counterparts. Recombinant allergens were shown to be useful tools in studying allergen specific B and T cell immune responses.

One way of recombinant allergen construction requires mRNA to be prepared from the tissue in question then reversibly transcribed into cDNA, which can then be used to build a cDNA library that can be screened with antibodies or DNA probes (Valenta and Kraft, 1995).

The first few attempts to produce recombinant Der p 1 in *S. cerevisiae* and *E. coli* were unsuccessful as the allergen was poorly expressed and had a weak IgE binding activity. Therefore, investigators tried finding a different expression system that could yield high concentration of protein secretion and would be easy to manipulate.

1.2.4 Expression of recombinant Der p 1 in *Pichia pastoris*:

It has become increasingly popular in recent years to use yeast as a cellular host for the expression of recombinant proteins. *Pichia* is easy to manipulate, faster and less

expensive to use than other eukaryotic expression systems, with the advantage of having higher expression levels and the ability to perform post-translational modifications like glycosylation (Cereghino and Cregg, 2000, Hamilton and Gerngross, 2007, Jacquet et al., 2002, Yokoyama, 2003)

Pichia pastoris needs methanol as a carbon source, which it oxidises to formaldehyde by an alcohol oxidase enzyme. Two genes in *Pichia* codes for alcohol oxidase *AOX1* and *AOX2*. The *AOX1* gene has been isolated and a plasmid containing the *AOX1* promotor is used to express the gene of interest. For the transcription of the desired protein, growth on glycerol with methanol induction is required (Cereghino and Cregg, 2000, Cereghino et al., 2002). Expression of protein in *Pichia* has the advantage of generating large amount of the desired protein in high concentrations with post-translational modifications like disulfide bonding and protein glycosylation. The desired protein is secreted in the culture media with low amounts of the yeast's own expressed proteins, using vectors such as pPICZalphaA that contains the AOX1 promoter that regulates methanol-induced expression of the gene of interest. This vector has also the alpha-factor secretion signal for secretion of the recombinant protein, a Zeocin

resistance gene for selection and a C-terminal peptide containing the c-myc epitope and a polyhistidine (6xHis) tag for detection and purification of the recombinant protein (Cereghino et al., 2002, Cereghino and Cregg, 2000).

Recently, several groups successfully produced recombinant forms of Der p 1 allergen expressed in *P. pastoris* with full enzymatic and IgE binding activity (Meno et al., 2005, de Halleux et al., 2006, Takai et al., 2005, van Oort et al., 2002). The advantages of using *P. pastoris* in expressing Der p 1 is that it leads to high level of secretion; this system is also feasible for fermentation to high cell density and can be scaled up without losing any yield (Jacquet et al., 2002). It is possible to modify the expressed recombinant Der p 1 to hyperglycosylated and hypoglycosylated forms using point mutations and enzymatic deglycosylation. The four possible glycosylation sites on Der p 1 are N16, N82, N132 and N 195; by mutating these sites the degree of glycosylation can be controlled in recombinant Der p 1 (Meno et al., 2005). In conclusion, this system is faster and less expensive than other eukaryotic systems and it produces enzymatically and immunogically active recombinant forms of Der p 1 (Jacquet et al., 2002, Meno et al., 2005).

1.2.5 Glycosylation in allergens:

Many allergens are glycosylated which raises the possibility that glycosyl groups may contribute to their allergenicity. Oligosaccharides are naturally added to proteins during synthesis in the ER and Golgi of the cells (Huby et al., 2000). Glycosylation is either N-linked where oligosaccharides are covalently attached to Asparagine, or they are O-linked to Serine/Threonine amino acids. This depends on which glycosylation motif is present within their primary amino acid sequence (Huby *et al.*, 2000, Fötisch and Vieths 2001).

The study of glycosylation in allergens began in the seventies when Bromelain, the protease from pineapple, was found to contain oligosaccharides with structural features that had not been found in mammalian glycoproteins, α1, 3-fucose and xylose (Bardor et al., 2003, Ishihara et al., 1979). These asparagine-linked carbohydrate moieties were found to be the most abundant in plants, insects and mammals but not in higher Apes such as humans, and therefore formed the structural basis of what is known as cross-reactive carbohydrate determinants (CCDs) (Commins and Platts-Mills, 2009, Altmann, 2007).

The link between CCDs and allergy was made after showing that α1,3-fucose and xylose are the most common carbohydrate epitopes recognised by human IgE antibodies. Studies have shown that up to 30% of allergic patients develop a specific anti-glycan IgE and that glycosylated epitopes make very good B cell epitopes (Mari, 2002, Commins and Platts-Mills, 2009, Calabozo et al., 2002, Okano et al., 1999, Petersen et al., 1998). Furthermore, it has been recently shown that IgE antibodies against CCD can produce clinically serious reactions specially in those reacting to mammalian galactose α1,3 galactose (Commins and Platts-Mills, 2009, Sandrine et al., 2009, Commins and Platts-Mills, 2010).

Glycosylated proteins can show higher uptake rates by DCs than their non-glycosylated counterparts. Hilmenyuk *et al* showed recently that glycation of ovalbumin increased its internalisation significantly by immature DCs and that it induced a Th2 immune response (Hilmenyuk et al., 2010, Anne et al., 2010). The cause of this phenomenon can be traced to the presence of lectin receptors like MR or DC-SIGN on DCs. This notion therefore supports the hypothesis that APCs recognise glycans on allergens efficiently and this

may eventually mediate an enhanced Th2 immune response (Fig 1-8).

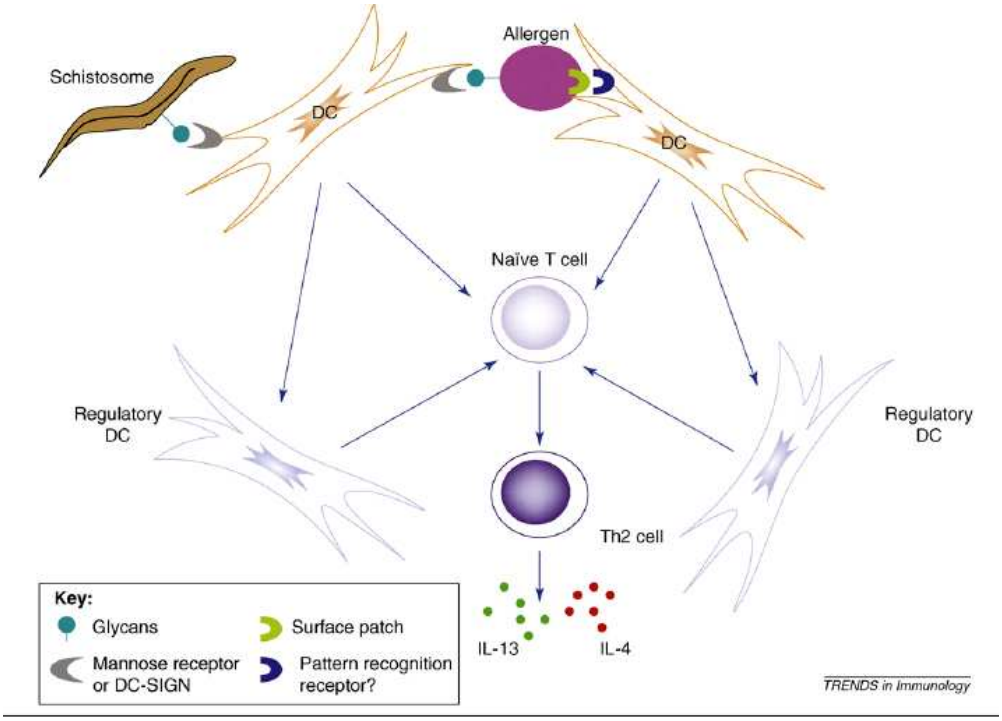


Fig 1-8: Surface sugar moieties on allergens bind allergen specific pattern recognition receptors like MR leading to Th2 responses (Shakib et al., 2008).

1.3 Aims of the project:

Much work was carried out over the years to understand the allergenicity of Der p 1 and its recognition by the immune system. However, the role of protein glycosylation in allergy has not been elucidated yet, but recent work in our lab has shown that the uptake of Der p 1 by the mannose receptor on dendritic cells is dependent on the level of Der p 1

glycosylation (Royer et al., 2010). The aim of this study is therefore to elucidate the role of glycosylation in Der p 1 recognition by the immune system, by producing different glycoforms of recombinant Der p 1 that could then be tested in dendritic cell uptake experiments.

Specific aims of the projects:

1. Investigate the pattern of glycosylation (quantity and quality) of major allergens from different sources including Der p 1.

2. Express Der p 1 in *P. pastoris* yeast cells to produce a recombinant form of Der p 1.

3. Produce different glyco-forms of recombinant Der p 1 using point mutation of specific glycosylation sites.

4. Produce different glyco-forms of recombinant Der p 1 using enzymatic and chemical deglycosylation.

5. Examine the allergenicity of these different glyco-forms of Der p 1 compared to their natural counterparts and assess their recognition by different cell surface receptors.

CHAPTER TWO: Investigating the pattern of glycosylation in allergens.

2.1 Introduction:

Allergens are foreign proteins that induce type I hypersensitivity reactions. In other words, they elicit Th2 immune responses, thus inducing IgE production and allergy. It has been suggested that what might differentiate allergens from other non-allergenic proteins lies in their protease activity, surface features and/or glycosylation patterns. These three features either singly or collectively might render some proteins allergenic (Shakib et al., 2008, Furmonaviciene et al., 2005, Huby et al., 2000). The protease activity and the peptide surface features of allergens have been discussed thoroughly in the literature (Russano *et al.*, 2008, Shakib *et al.*, 2008, Wills-Karp *et al.*, 2009). The glycosylation pattern of allergens, however, is only now beginning to get attention as possible distinguishing features of these proteins. Much of the research in this area has so far only dealt with the determination of the carbohydrate content of allergens, without much consideration for the whole carbohydrate structure and the pattern of glycosylation (Poltl *et al.*, 2007, Fötisch and Vieths 2001, Altmann, 2007).

The role of carbohydrates in allergy has been discussed in recent years and remains controversial (Altmann, 2007, Fötisch and Vieths 2001, Paschinger et al., 2005, Okano et al., 1999). It is well known that carbohydrate determinants are the most frequently encountered epitope structures for IgE (Fötisch and Vieths 2001, Chunsheng et al., 2008), and as such have been named Cross-reactive Carbohydrate Determinants (CCD). These determinants are asparagine linked carbohydrate moieties and they mainly consist of xylose and core-3-linked fucose which forms the vital part of two independent IgE epitopes (Chunsheng et al., 2008). These CCDs are mainly found in plants, insects and parasites, but are absent in mammals and are therefore immunogenic (Paschinger et al., 2005, Altmann, 2007)

In an attempt to have a better understanding of the role of carbohydrates in allergy, the most commonly encountered allergens were examined in this chapter for their carbohydrate content by using labelled lectins that react to specific sugar moieties (Table 2-1). Defining the quantity and quality of glycosylation in allergens is a major first step towards demonstrating a link between carbohydrates and allergen recognition by the innate immune system. Most of the previous research on allergen glycosylation concentrated on the presence of xylose and fucose (Altmann, 2007, Chunsheng et

al., 2008). In this work, the detection of 1,2 1,3 and 1,6 mannose was done using the lectin GNA that reacts with high mannose structures on proteins. It was also possible to identify 1,3 fucose in allergens via a specific antibody. Examining O-glycosylation in allergens was done using MAA, SNA, PNA and DSA which collectively recognise 1,3 and 1,4 galactose in addition to sialic acid. Collectively, these antibodies helped to define the prevalence of different glycan moieties in allergens.

2.2 Materials and methods:

2.2.1 Allergen and protein sample preparation:

Purified natural and recombinant Der p 1, Der p 2, Fel d 1, deglycosylated Fel d 1 (missing a major glycosylation site), Can f 1, Ara h 1, Bla g 2 and Ber e 1 were bought from Indoor Biotechnology UK. Papain and Bromelain were bought from Sigma. Non-allergens Staphopain B (Biocentrum ltd, UK), Calpain ll, Cathepsin B and C were purchased from Sigma. CPB was kindly provided by Prof Jeremy Mottram, University of Glasgow.

2.2.2 SDS polyacrylamide gel electrophoresis (SDS- PAGE):

All protein preparations were analysed by electrophoresis using 12% Novex Tris-Glycine precast gels from Invitrogen. Tris Glycine running buffer (TGS) was prepared (30.3g Tris, 144g Glycine and 10g SDS made up to 1 L with water) and protein

samples were run in XCell *SureLock*™ Mini-Cell from Invitrogen under reducing conditions for 1hr 30mins at 125V. Samples were diluted with 4X sample buffer (0.25M Tris-HCl pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue and 0.3M DTT) and heated to 95°C for 5 mins prior to loading. Protein concentrations upon loading were 5µg per well; 5µl of a control pre-stained protein ladder (Fermentas) was added to track sample migration in the first lane of the gel.

2.2.3 Western blot analysis:

After gel electrophoresis, nitrocellulose membranes (Amersham, UK) were cut, soaked in Tris Glycine (TG blotting buffer (25mM Tris pH8.5, 0.2M Glycine and 20% Methanol), gel and membrane were assembled in an Invitrogen XCell II™ Blot Module apparatus and soaked with 60 ml of TG blotting buffer. The gels were then transferred for 1 hr at 30V. When finished, the membranes were blocked overnight with 5% BSA in TBS (0.05 M Tris-HCl, 0.15 M NaCl at pH 7.5) at 4°C. In the glycan detection process, the DIG Glycan Differentiation Kit (from Roche Applied Sciences, Germany) was used. The membranes were washed twice with TBS and once with buffer 1 (TBS; 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ at pH 7.5). The required amount of lectin solution was added (for GNA, SNA and DSA 10 µl each; for MAA 50 µl and for PNA 100 µl) to 10 ml of buffer 1. All these lectins that are Digoxigenin (DIG

labelled were incubated separately for 1 hr with the membranes and the membranes were then washed three times with TBS to remove any unbound antibodies. The secondary antibody was prepared (10 µl of anti-DIG conjugated to Alkaline phosphatase (diluted in 10 ml of TBS) and the membranes were incubated with the antibody for one hour (Fig 2-1). The detection solution was prepared by using 10 ml Buffer 2 (0.1 M Tris-HCl, 0.05 M MgC_2, 0.1 M NaCl at pH 9.5) diluted in 200 µl NBT/BCIP solution and the membranes were then incubated with the detection solution for 20 mins in the dark (Colorimetric detection). The reaction was stopped by washing with ultra pure H_2O. Membranes were scanned using BioRad GS800 Densitometer. Positive and negative controls are provided with the DIG Glycan detection kit and were used in the western blot experiments.

Table 2-1: The Lectins used to detect glycosylation in allergens. All lectins were purchased from Roche Applied Science, Germany.

Labeled lectin	Detection
GNA,Digoxigenin-labeled (Galanthus nivalis agglutinin)	Recognizes terminal mannose, (1–3), (1–6) or (1-2) linked to mannose
SNA,Digoxigenin labeled(Sambucus nigra agglutinin)	Recognizes sialic acid linked (2–6) to galactose
MAA, Digoxigenin-labeled (Maackia amurensis agglutinin)	Recognizes sialic acid linked (2–3) to galactose
PNA,Digoxigenin-Labeled (Peanut agglutinin) PNA,Digoxigenin-Labeled	Recognizes the core disaccharide galactose (1–3) N acetylgalactosamine
DSA, Digoxigenin-Labeled (Datura stramonium agglutinin)	Recognizes Gal-(1–4)

The detection of 1,3 fucose was done using anti-1,3 fucose rabbit polyclonal antibody (no. AS07 268, Agrisera, UK). This antibody cross-reacts with fucose residues bound to N-Glycans in alpha 1,3 in plants and insects. The concentration of antibody used was 1µg/10ml of TBS buffer; the reaction mixture was incubated for one hour then washed three times with TBS buffer. Anti-rabbit antibody conjugated with horseradish peroxidise (Sigma) was used as a secondary antibody, again incubated for one hour, washed and the reaction was detected using ECL chemiluminescent detection reagent (GE Healthcare Life Sciences) according to

manufacturer's protocol**.** Membranes were scanned using BioRad GS800 Densitometer. Bromelain was used as a positive control and Cathepsin from mammalian origin was used as a negative control

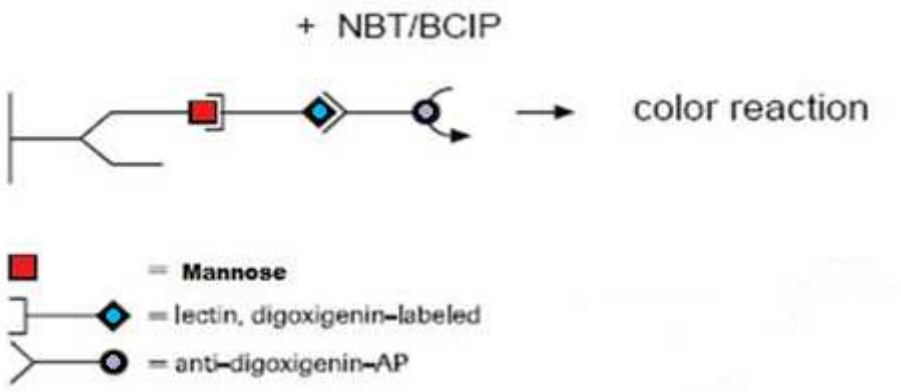


Fig 2-1: The principle of Colorimetric detection of carbohydrates on allergens by Lectin binding.

2.2.4 Prediction of glycan structures in allergens:

The oligosaccharide structures that are shown in this chapter are symbolic representation suggested by the Consortium of Functional Glycomics (www.functionalglycomics.org).

The structure codes comply with the proglycan system (www.proglycan.com).

2.3 Results:

2.3.1 Detecting the pattern of N- and O-glycosylation in allergens:

By using GNA, SNA, PNA, MMA and DSA labelled lectins and anti-1,3 fucose antibody, allergens were scanned for the different sugar moieties they possess. Der p 1, a cysteine protease allergen from *Dermatophagoides pteronyssinus*, reacted with GNA which recognises 1,2 1,3 and 1,6 mannose, suggesting that it has high mannose N-glycans in its native form (Fig 2-1 A). Der p 1 also showed a positive reaction with 1,3 fucose (Table 2-2), which indicates that it has part of the CCD 1,3 fucose on its N-glycosylation site which is linked to asparagine. It also reacted with DSA, PNA and SNA, which respectively recognise 1,4 galactose, 1,3 galactose and sialic acid linked 2-6 to galactose (Fig 2-2 A). Der p 1 failed to react with MMA, thus suggesting that it does not contain any sialic acid binding 2-3 to galactose.

When examining Papain, a cysteine protease allergen from papaya, and Bromelain, a cysteine protease allergen from pineapple, their glycosylation pattern was found to be similar to that of Der p 1, and mannosylation seems to be the dominant positive reaction in all these cysteine protease allergens (Fig 2-2 B & D).

The recombinant preparation of Der p 1 that is produced in *Pichia pastoris* reacted with GNA to a higher degree than the native preparation (Fig 2-2 C). The band itself was diffuse, suggesting hyperglycosylation and its positive reaction with GNA confirmed that most of the glycosylation is due to mannosylation which is expected as proteins expressed in yeast tend to be hypermannosylated (Jacquet et al., 2002, Cereghino and Cregg, 2000, van Oort et al., 2002, Meno et al., 2005, Staudacher et al., 1999). The preparation also reacted with PNA suggesting that it also has some O-glycosylation present as 1,3 galactose. The recombinant preparation does not have any sialic acid or 1,4 galactose (Fig 2-2 C).

Fel d 1, the cat allergen *Felis domesticus*, is shown here to have low levels of mannan with strong reactions also with DSA and MAA, thus suggesting that it has 1,4 galactose and sialic acid (Table 2-2). It does not, however, contain 1,3 fucose which is expected as 1,3 fucose is not present in mammals (Fig 2-8 A). Its deglycosylated counterpart does not contain any mannan, but it does contain sialic acid and 1,3 galactose (Table 2-2). Can F 1 , the dog allergen *Canis familiaris*, is also not fucosylated as it is from a mammalian source (Staudacher et al., 1999, Altmann, 2007) (Fig 2-8 A), but it is mannosylated and appears to contain sialic acid (Table 2-2). The Brazilian nut allergen, Ber e 1, is mannosylated also and

fucosylated (Table 2-2). The two allergens that showed a very strong reaction with mannan, in addition to rDer p 1, were Ara h 1 (*Arachis hypogaea*) and Bla g 2 (*Blattella germanica*). These two allergens are highly mannosylated in their native form which is indicated in Table 2-2.

Other major allergens such as Der p 2 failed to give a positive reaction with GNA, indicating the lack of mannan in this allergen (Fig 2-2 E). It did, however, react with 1,3 fucose (Fig 2-8 B) and also reacted positively with DSA, MMA, PNA and SNA, which suggests that although lacking mannan, Der p 2 is still glycosylated.

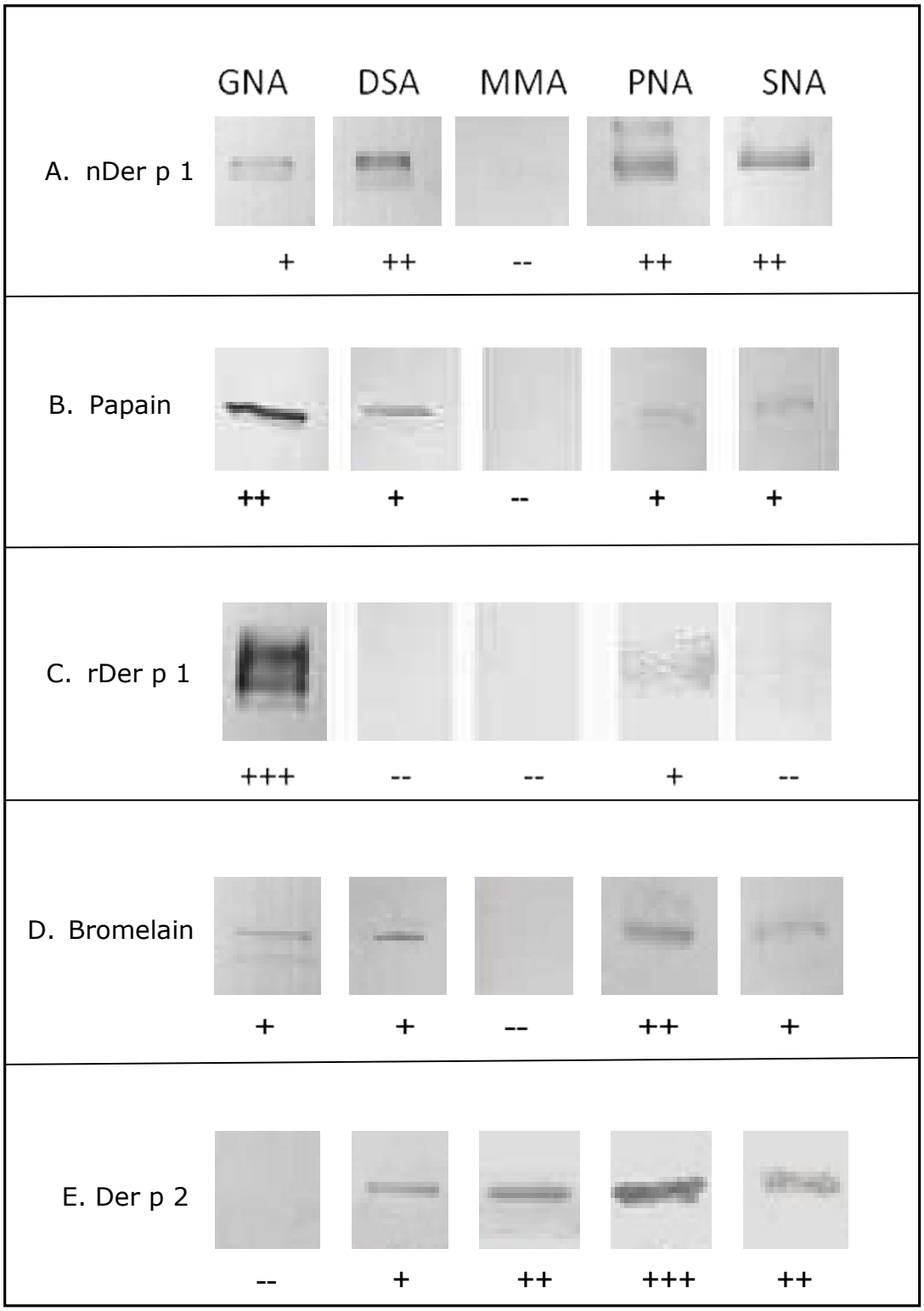


Fig 2-2. Western blot analysis using different lectins that recognise specific carbohydrate structures on allergens. Anti-GNA (recognises terminal mannose

(1–3), (1–6) or (1–2) linked to mannose, anti-DSA recognises Gal-(1–4)GlcNAc in complex, anti-MMA recognises sialic acid linked (2–3) to galactosee, anti-PNA recognises the core disaccharide galactose (1–3) and anti-SNA recognises sialic acid linked (2–6) to galactose. +++: strong reaction, ++: moderate reaction, +: mild, --: no reaction, number of independent experiments ≥3.

Table 2-2: The table below shows the western blot results of different lectin reactions with different allergens.

Allergen	GNA (anti mannose)	DSA	MMA	PNA	SNA	Anti-1,3 Fucose
nFel d 1	+	+	+++	-	-	-
DFel d 1	-	-	+	++	-	-
Derp2	-	+	++	+++	++	+
Ara h 1	+++	-	+	++	++	++
Bla g 2	+++	++	+	++	++	+++
Can f 1	+	-	+	-	++	-
Ber e 1	+	-	++	+	++	+
rDer p 1	+++	-	-	+	-	++
Der p 1	+	++	--	++	++	+++
Papain	++	+	-	+	+	++
Bromelain	+	+	-	++	+	+++

+++: strong reaction, ++: moderate reaction, +: mild reaction, --: no reaction, number of independent experiments ≥3.

2.3.2 Comparative studies of allergen and non-allergen glycosylation patterns:

As mannosylation appears to be the dominant glycosylation among allergens (Table 2-2), comparative carbohydrate analysis was done for proteins that are not known to elicit

allergic immune responses, yet share the same protein family with allergens. The cysteine protease family includes Der p 1, Papain and Bromelain as allergens and Staphopain B, Calpain and Cysteine Protease B (CPB) as non-allergens (Bühling et al., 2002, Smagur et al., 2009, Goll et al., 2003, Ghaemmaghami et al., 2002, Oliver Schulz, 1998). All these proteins have potent cysteine protease activity, but nothing is known about their glycosylation pattern. Following experiments using GNA lectin it became clear that the non-allergens Staphopain B and Calpain do not react with GNA, thus indicating that they are not mannosylated like allergens (Fig 2-3). Cysteine protease B showed a very weak reaction with GNA, again indicating that it has only a minimal amount of mannose moities. Staphopain B also did not react with any of the other lectins, indicating that it does not have any O-glycosylation. It did, however, give a weak reaction with the 1,3 fucose antibody (Fig 2-9 B). Calpain and CPB did react with PNA, MMA, SNA and DSA (Figs 2-4, 2-5, 2-6, 2-7), indicating the presence of O-glycosylation. These two proteins also gave a weak positive reaction with 1,3 fucose (Fig 2-9 B).

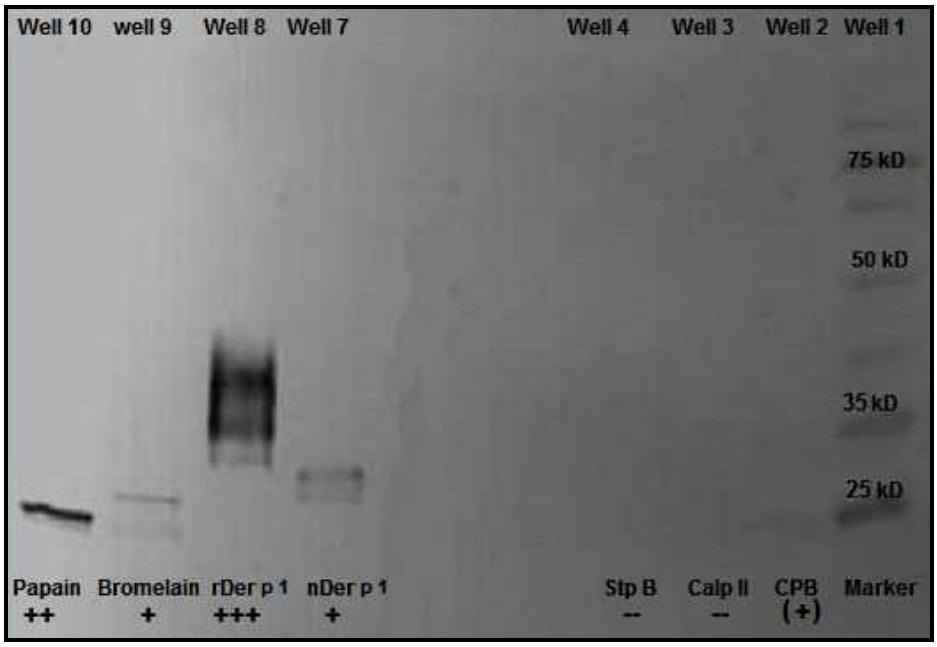


Fig 2-3: Immunoblot of a cysteine protease family against GNA (*Galanthus nivalis* agglutinin) which recognises terminal mannose, (1–3), (1–6) or (1–2) linked to mannose. Cysteine proteases that are allergens: rDerp1, nDerp1, Bromalain and Papain. Cysteine proteases that are non-allergens: Calpain II and Cysteine protease B (CPB). Cysteine proteases that induce innate immunity: Staphopain B. 5μgs of the protein is loaded in each well. +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=2.

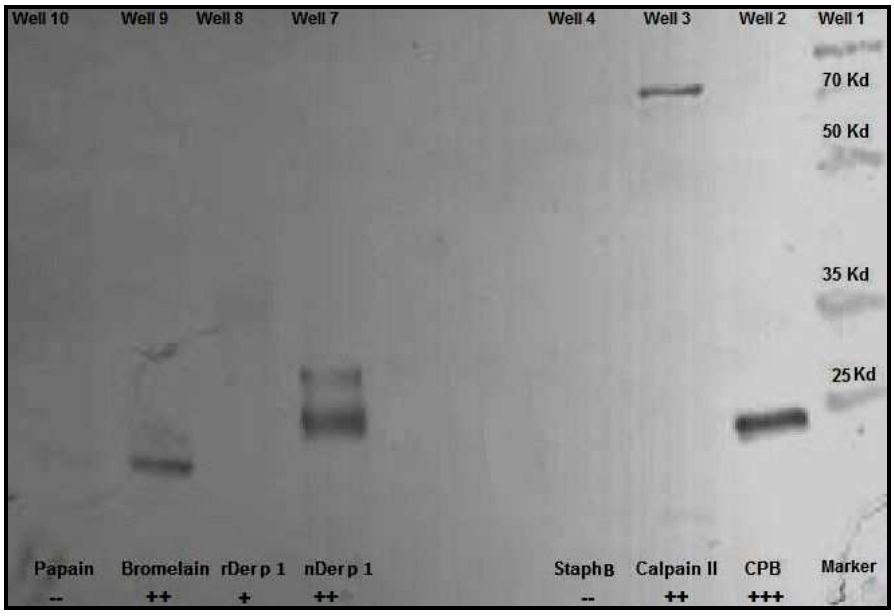


Fig 2-4: Immunoblot of a cysteine protease family against PNA (Peanut agglutinin), which recognises the core disaccharide galactose (1–3) N-acetylgalactosamine. +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=2.

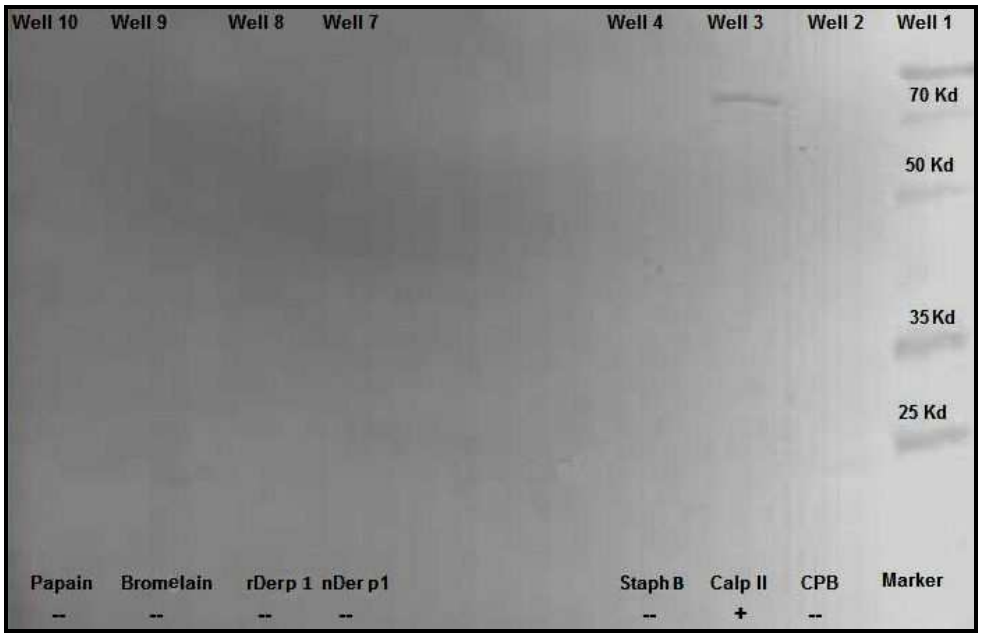


Fig 2-5: Immunoblot of a cysteine protease family against MMA (Maackia *amurensis* agglutinin), which recognises sialic acid linked (2–3) to galactose. +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=2.

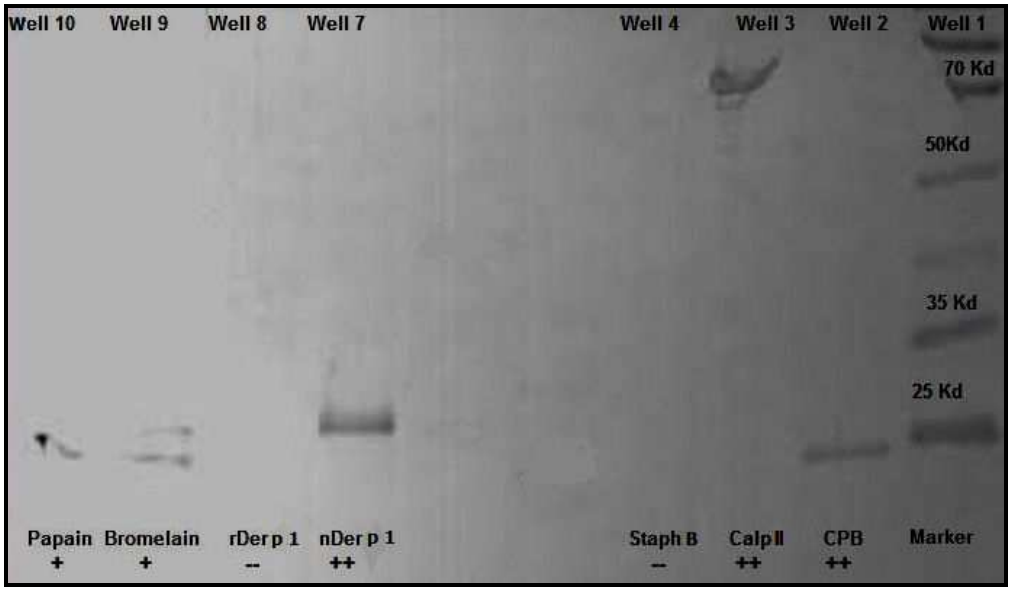


Fig 2-6: Immunoblot of a cysteine protease family against SNA (*Sambucus nigra* agglutinin), which recognises sialic acid linked (2–6) to galactose, thus in combination with the data from lectin MMA which recognises sialic acid linked 2,3 to galactose (Fig 2-5), SNA is suitable for identifying sialylated glycan chains linked 2,6 to galactose on proteins. +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=2.

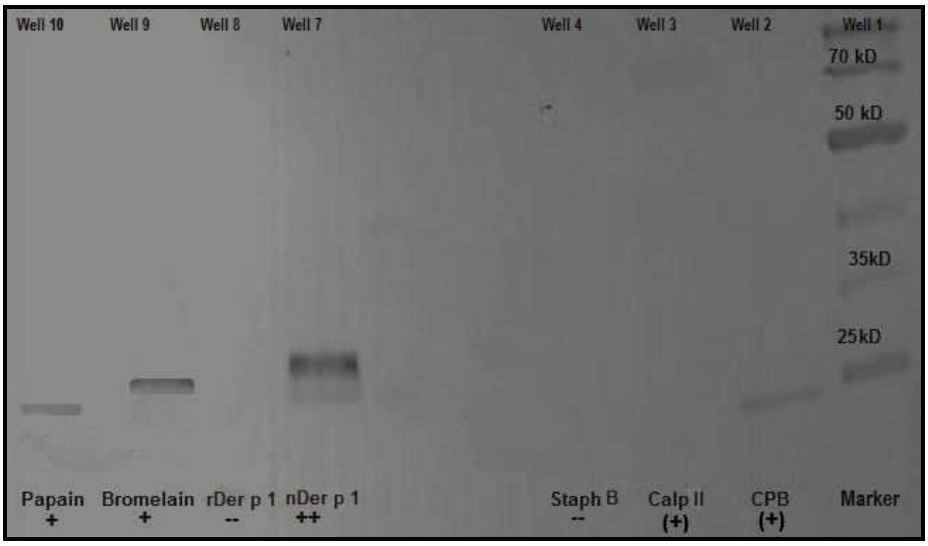
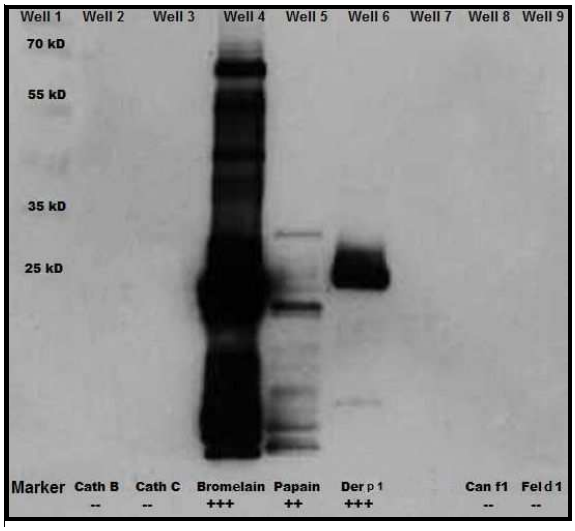


Fig 2-7: Immunoblot of a cysteine protease family against DSA (*Datura stramonium* agglutinin), which recognises Gal-(1–4) GlcNAc in complex and hybrid N-glycans in O-glycans and GlcNAc in O-glycans. +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=2.

A.



B.

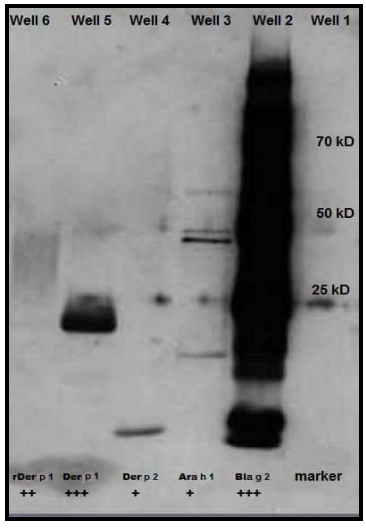


Fig 2-8: Allergen and non-allergen reactions with fucose 1, 3 antibody. Der p 1, rDer p 1, Bromelain, Papain, Ara h 1, Bla g 2 and Der p 2 are fucosylated, but allergens derived from mammalian sources like Can f 1 and Fel d 1 are not. The non-allergens cathepsin B and C are not fucosylated (derived from bovine origin). +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=3.

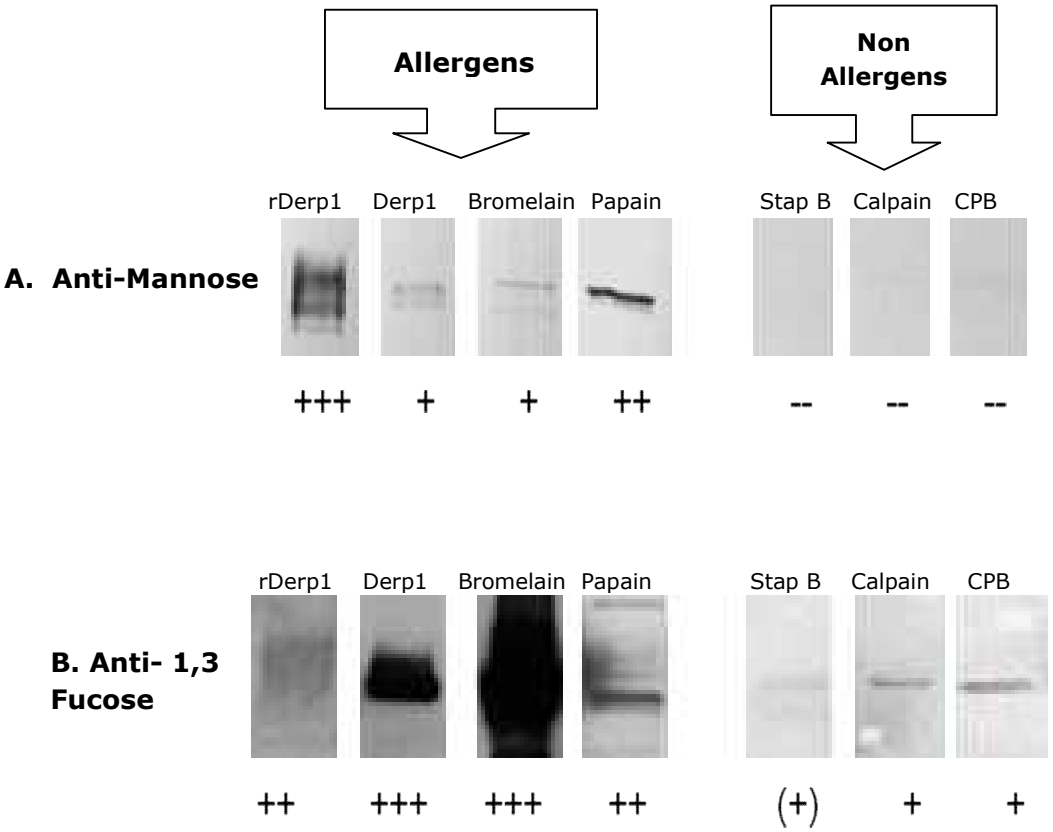


Fig 2-9: Comparative analysis between allergens and non-allergens, that are cysteine proteases, in term of mannosylation and fucosylation. Allergens are strongly mannosylated and have stronger reaction with 1,3 fucose compared to non-allergens. +++: strong reaction, ++: moderate reaction, +: mild reaction, (+): weak reaction, --: no reaction, number of independent experiments=2.

2.3.3 Speculated structure of carbohydrates on allergens:

The N-glycosylation structures in allergens were predicted with the help of a symbolic representation suggested by the Consortium of Functional Glycomics (Fig 2-10). The asparagine linked structure is known to contain N-Acetylglucosamine (GlcNAc). Data from this chapter showed that 1, 3 fucose is present in Der p 1, in addition to 1, 3 and 1, 6 mannose (Fig 2-10 A). In the literature, xylose was shown to be present in Der p 1 and this information, together with those presented earlier, would make it possible to predict how the sugar moieties are arranged in this allergen. The recombinant Der p 1 preparation reacted with fucose and mannose, and gave a stronger reaction with high mannose, so it can be speculated that the branching of mannose would be different than for native Der p 1. As rDer p 1 is expressed in yeast, it is highly likely that it contains 1,2, 1,3 and 1,6 mannose (Fig 2-10 A). The higher branching of mannose is expected to be 1,2 (Vervecken et al., 2007) The high mannosylation of Ara h 1 and Bla g 2 is expressed as more branching and the presence of 1,3 fucose is stated (Fig 2-10 C). The structure of Papain and Bromelain is already described in the literature, and their mannosylation and fucylation was confirmed by western blot analysis (Fig 2-10 B). What we know at the moment about the N-glycosylation

of non-allergens is that they are not mannosylated which is shown in Fig 2-10 (F). The fact that mammalian proteins do not possess 1,3 fucose is shown also in Fig 2-10 (D & G).

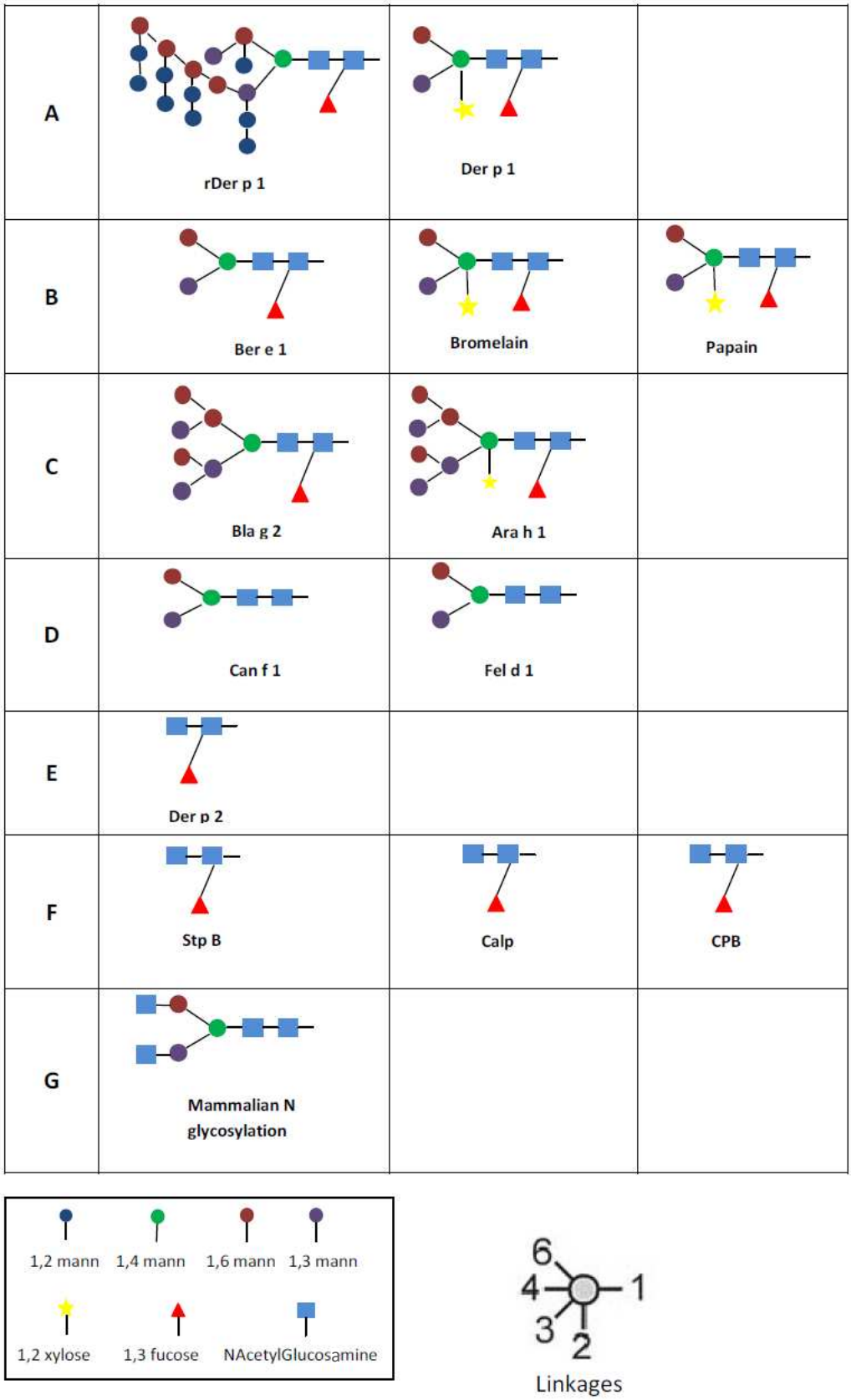


Fig 2-10: A speculated structure of N-glycosylation in some allergens compared to mammalian N-glycosylation. Linkages and legends are explained in the figure.

2.4 Discussion:

Glycosylation in allergens is a key feature and mannosylation seems to be the dominant glycosylation pattern with the exception of Der p 2. Reports on glycosylation in *Pichia* expressed proteins indicated that most of these proteins are highly mannosylated (Cereghino and Cregg, 2000, Cereghino et al., 2002, Meno et al., 2005), which is what this chapter confirmed by using a GNA lectin that specifically binds to high mannose structures. This work also demonstrated that Der p 1 is mannosylated in its natural form, which confirms what has already been described in the literature using other methods of glycan identification (Yang et al., 2008). In addition to Der p 1 we have also shown the predominance of mannan in the glycosylation of other environmental allergens Ara h 1, Bla g 1, Ber e 1, Can f 1, Fel d 1, Bromelain and Papain. Other groups reported the presence of mannan in Cedar Allergen Cry j 1 (Aoki et al., 2010), pollen allergen Cha o 1 (Kimura Y, 2008), yellow jacket allergen Ves v 2 (Seppälä et al., 2009) and Ovalbumin (Plasencia et al., 2008). Ara h 1, Cor a 11, Jug r 2 and Ana o 1 have been reported to contain a xylose and mannose in the *N*-glycan chain (Lauer, 2004, Kolarich and Altmann, 2000). Fucose 1,3 is reported to be present in a wide range of allergens like Hev b 1, Ara h 1, Bromelain and Papain (Malik et al., 2008, van Ree et al., 2000, Altmann, 2007), and

in this work we demonstrated for the first time that Der p 1, rDer p 1 and Der p 2 contain fucose 1,3. This is important in understanding the pattern of N-glycosylation in allergens, as fucose 1,3 is not present in mammalian cells and as such is considered non-self to humans. There is one report mentioning the finding of xylose in Der p 1, which represents another evidence for the presence of CCD in Der p 1 (Yang *et al.*, 2008)(Table 2-3). Cat (Fel d 1) and dog (Can f 1) allergens did not have fucose 1,3 antibody which is expected as these two allergens are derived from mammalian sources. In this context, a speculative structure for N-glycosylation in different allergens was depicted by combining the results of different lectin antibody reactions with what has already been described in literature regarding allergen N-glycosylation. A pattern has emerged with major differences between allergens and their non-allergen counterparts. The quality and quantity of mannosylation and fucosylation between allergens like Der p 1, Papain, Bromelain, Bla g 2 and Ara h 1 and non-allergens like Cathepsin B, Cathepsin C, CPB, Calpain and Staphopain B is different. A conclusion can be made that non-allergens possess a very low quantity of mannose and fucose 1, 3. Previous reports also mention that xylose 1,2 and xylose 1,3 are present in some allergens like Papain, Ara h 1 and Bromelain (Altmann, 2007, Shreffler et al., 2006, van Ree et al., 2000).

The notion that Der p 1 and Der p 2 possess 1,3 fucose in their native form provides a better understanding of their N-glycosylation pattern.

The detection of galactose 1,3 galactose 1,4 and sialic acid in allergens gave a better understanding of O-glycosylation in allergens. Although most reports concentrate on N-glycosylation as a target for lectin receptors on antigen presenting cells, some recent reports did suggest that O-glycosylation by itself plays a role in CCD (Commins and Platts-Mills, 2010, Sandrine *et al.*, 2009, Leonard R, 2005), which is why it is important to know the specific structures of O-glycans in allergens.

Table 2-3. N-glycans detected in Der p 1 (Yang et al., 2008).

Relative abundances of N-glycans detected in Der p 1

Fraction		m/z $(M + Na)^+$		Glycoform	Ratio (%)
		MALDI-TOF-MS	Calculated		
1		1821.56	1821.64	$Man_8GlcNAc_2$	26.3
2		1983.39	1983.78	$Man_9GlcNAc_2$	12.9
4	a	1659.36	1659.50	$Man_7GlcNAc_2$	1.6
	c	1983.03	1983.78	$Glu_1Man_8GlcNAc_2$	26.8
5	b	2145.26	2145.92	$Glu_1Man_9GlcNAc_2$	25.4
6	b	1143.90	1143.05	$Man_3XylGlcNAc_2$	4.9
	e	1335.40	1335.22	$Man_5GlcNAc_2$	2.1

CHAPTER THREE: The expression of Der p 1 allergen in *Pichia pastoris*.

3.1 Introduction:

Der p 1 is a 25 kDa allergen with cysteine protease activity. It is produced in the mid gut of the mite and found in mite faecal pellets. It has been shown that up to 90% of individuals who are sensitized to house dust mite allergens are actually responsive to Der p 1 (Thomas and Smith, 1998, Burtin et al., 2009, Richardson et al., 2005, Custovic et al., 1996, Spieksma FT, 1967).The molecular structure of Der p 1 has been the subject of many studies. The gene encoding Der p 1 has been cloned and sequenced, and the cDNA clone coding for Der p 1 was first sequenced by Chua *et al.,* (1988). It codes for a 222 residue mature protein having a molecular weight of 25,371 and containing four potential *N*-glycosylation sites. Der p 1 is produced in the mite as enzymaticaly inactive proenzyme that becomes active after cleavage of the propeptide. When Der p 1 is extracted from the mite faeces it is in the mature form (nDer p 1) (van Oort et al., 2002, Chua et al., 1988, Meno et al., 2005, de Halleux et al., 2006). Meno et al (2005) were able to produce a recombinant form of Der p 1 that is hypoglycosylated

using an N132E mutation that rendered the protein non-susceptible to glycosylation at this site. The recombinant form was expressed in *Pichia pastoris*.

Several groups produced recombinant forms for Der p 1 allergen expressed in *P. pastoris* with full enzymatic and IgE binding activity (Meno et al., 2005, de Halleux et al., 2006, Takai et al., 2005, van Oort et al., 2002). The advantages of using *P. pastoris* in expressing Der p1 is that it leads to a high level of secretion; this system is also easy for fermentation to high cell density and can be scaled up without losing any yield (Jacquet et al., 2002). More importantly, this system lends itself to modification of the expressed recombinant Der p 1 to produce hyperglycosylated and hypoglycosylated forms using point mutations and enzymatic deglycosylation. The four possible glycosylation sites on Der p 1 are N16, N82, N132 and N 195; by mutating these sites the degree of glycosylation can be controlled in recombinant Der p 1 (Meno et al., 2005).

Recent work in our lab has shown that the uptake of Der p 1 by the mannose receptor on dendritic cells is dependent on the level of Der p 1 glycosylation. This clearly raises the question of the role of glycans in allergen recognition and uptake by dendritic cells, we therefore aimed to produce different

glycoforms of Der p 1 through expressing it in *Pichia pastoris,* which is reported to express Der p 1 in a hypermannosylated form (Meno et al., 2005, de Halleux et al., 2006, Takai et al., 2005, van Oort et al., 2002). We were also aiming to produce different glyco-forms of Der p 1 that can be tested for allergenicity.

3.2 Materials and Methods:

3.2.1 cDNA purification from λ phage 10 and λ phage 11 libraries:

λ phage 10 and λ phage 11 libraries (kindly provided by W. R. Thomas, Princess Margaret Children's Medical Research Foundation, Perth, Australia) were used to obtain pro and mature Der p 1 sequences. First, phenol/chloroform extraction was done for both libraries to purify the cDNA which will partition in to the aqueous layer and extract protein components in to the organic phase.

The concentration of the cDNA for both libraries was measured using the nanodrop ND 1000 spectrophotometer. In λ phage 10 the concentration for cDNA was 4101.3 ng/µl and in λ phage 11 the concentration for cDNA was 3781.4 ng/µl.

3.2.2 Cloning of Pro and Mature Der p 1:

From the λ phage 10 and 11 library of the house dust mite *Dermatophagoides pteroyssinus* pro and mature Der p 1 cDNA sequences products were amplified using Polymerase Chain

Reaction (PCR). The reference for the primers used in amplifying Der p 1 was van Oort *et al.*, 2002. To facilitate cloning and ligation, KpnI restriction enzyme cutting site (GGT ACC) was introduced to the 3' primer and XhoI 5' restriction enzyme cutting site (GAG CTC) was introduced to the 5' primer (Table 3-1).

It was noticed that the pPICZα A plasmid has a chain termination sequence (**TAA**) that was introduced consequently to the 3' primer. In order to make sure that the expression does not stop before the C-terminal polyhistidine tag, as this will help in purifying Der p 1 recombinant protein after expression, a mutation was introduced to primer 4n.

PCR amplification for pro Der p 1 was done using primers (4n+p1) and (4s+p1) (Table 3-1), while PCR amplification for mature Der p 1 was done using primers (4n+p3) and (4s+p3) (Table 3-1). The PCR reaction was carried out in a volume of 25 µl containing 1 µg of cDNA template. The reaction conditions were as follow: initial denaturation at 94°C for 10 mins followed by 5 cycles of denaturing at 94°C for 90 mins, annealing at 50°C for 90 mins and extention at 72°C for 4 mins. Then another 5 cycles of denaturating at 94°C for 90 mins, annealing at 55°C for 90 mins and extention at 72°C for 4 mins and a final 25 cycles of denaturing at 94°C for 90 mins, annealing at 60°C for 90 mins and extention at 72°C for 4 mins.The reaction final extension

was at 72°C for 20 mins.Then they were run on 0.8% e. gels for detection.

Table 3-1: Primers used to obtain Der p 1 cDNA by PCR.

3' primers	
To amplify mature and pro Der p 1 with kpnI restriction digestion (with no stop codon) (4n)	5'GGG GGT ACC TTG AGA ATG ACA ACA TAT GG 3'
To amplify mature and pro Der p 1 with kpnI restriction digestion (with a stop codon) (4s)	5'GGG GGT ACC **TTA** GAG AAT GAC AAC ATA TGG 3'
To amplify mature and pro Der p 1 with xhol restriction digestion site (with a stop codon) (p2)	5' GGG GAG CTC **TTA** GAG AAT GAC AAC ATA TGG 3'

5' primers	
To amplify pro Der p 1 with xhol restriction digestion (p1)	5'GGG CTC GAG AAA AGA CGT CCA TCA TCG ATC AAA ACT TTT G 3'
To amplify mature Der p 1 with xhol restriction digestion (p3)	5' GGG CTC GAG AAA AGA CGT CCA TCA TCG ATC AAA ACT ATG 3'

Table 3-2: Primers used for sequencing (from Invitrogen).

Primer	Sequence
5´ *AOX1* sequencing primer (5´ *Pichia* primer)	5´-GACTGGTTCCAATTGACAAGC-3´
3´ *AOX1* sequencing primer (3´ *Pichia* primer)	5´-GCAAATGGCATTCTGACATCC-3´
α-Factor sequencing primer	5´-TACTATTGCCAGCATTGCTGC-3´

3.2.3 Purification of Der p 1 PCR product:

The PCR products for the pro and mature Der p 1 were purified using QIAquick PCR purification kit according to manufacturer protocol, and its concentrations were measured using the nanodrop ND 1000 spectrophotometer, then the product was run on agarose 2% e-gel for detection.

3.2.4 Sequencing mature and pro Der p 1 PCR product:

Sequencing reaction using Big Dye v1.1 was done according to the following conditions: denaturing at 96°C for 30 sec, annealing at 50°C for 15 sec and extention at 60°C for 4 mins, then a final extention at 28°C for 1min.

Then samples were precipitated by ethanol precipitation and sequenced using the ABI 3130 analyser to confirm that the PCR products are for the cDNA of mature and pro Der p 1. Comparison alignment for the amplified PCR product of the mature and pro Der p 1 with gene bank data for the complete Der p 1 allergen was done using the Blast search engine to detect compatibility.

3.2.5 Transformation of pro and mature Der p 1 insert in to TOP 10 *E. coli* cells:

Double restriction enzyme digest reaction was carried out for both pPICZ α A plasmid and Der p 1 inserts using Kpn I and Xho I restriction digest enzymes (from Invitrogen). For Der p 1 inserts 10 U of Kpn I were used in the first digest with 2.5 µl REact® 4 enzyme buffer from Invitrogen and up to 0.49ug of the Der p 1 DNA product then distilled water was added to have a final reaction mixture of 25 µl. This mixture was incubated at 37°C for 1 hour then deactivated at 80°C for 20 mins. After that another digest using 10 U of Xho l was carried out for another 1 hour at 37°C. The same conditions were used to digest 0.5µg of pPICZ α A plasmid. After deactivation of the reaction at 80°C both the plasmid and Der p 1 inserts were purified using Quiagen purification kit and its concentrations were measured using the nanodrop ND 1000 spectrophotometer. Then, the PCR product of pro and mature Der p 1 was ligated to pPICZ α A

Vector using Ready-To-Go T4 DNA ligase kit according to manufacturer protocol. Different molar ratios (1:4, 1:7, 1:10 and 1:20) for vector against insert were used to optimise the ligation reaction. Then TOP 10 *E. coli* chemically competent cells (from Invitrogen) were used to transform pPICZ α A vector. Competent cells were kept chilled on ice during transformation, up to 35.0 ng of DNA (Plasmid with Insert) in 10 µl volume was mixed with 80 µl competent cells. For the positive control 9.0 ng of pPICZ α A was mixed with 80 µl competent cells while for negative control no vector was added to the competent cells. All mixtures were immediately placed on ice for 30 minutes then they were heat shocked at 42°C for 45 sec then 900 µl of LB media was added to the mixtures and incubated for 1 hr at 37°C. The reaction was then plated on LB Zeocin plates overnight at 37°C. Colonies that grew overnight were detected by PCR using the α-Factor sequencing primer and 3´ *AOX1* sequencing primer to check for the plasmid. The PCR reaction conditions were as follow: initial denaturation at 94°C for 10 mins followed by 35 cycles of denaturing at 94°C for 90 mins, annealing at 54°C for 90 mins and extension at 72°C for 2 mins and a final extension at 72°C for 2 mins. The plasmid used was purified from the *E. coli* culture using PurLink Quick Plasmid Miniprep kit according to manufacturer protocol, then it was sequenced using Big Dye v1.1 sequencing reaction as follow:

denaturing at 96°C for 30 sec, annealing at 50°C for 15 sec and extention at 60°C for 4 mins for 25 cycles, then a final extention at 28°C for 1min. Only colonies having the Der p 1 insert ligated with the plasmid were selected, streaked and then grown in LB Zeocin culture overnight at 37°C.

3.2.6 Transformation of pro and mature Der p 1 insert in to X33 yeast competent cells:

Competent cells from X33 yeast cell culture (provided in the easyComp kit from invitrogen) were prepared acoording to manufacturer protocol.The plasmid used was linearized using SacI restriction digest at 37°C for 2 hours using 5.0 U of Sac I to digest 5 µg of plasmid DNA. Buffer 1 was added to the reaction provided from New England BioLabs. Then up to 5 µg of the linearized plasmid was transferred to *Pichia pastoris* X33 competent cells using the EasyComp transformation kit from invitrogen according to the kit protocol. Positive and negative controls were introduced during transformation reaction. After that *Pichia* cells were plated on YPD Zeocin containg media at 30°C for 2 days and the colonies that were growing were detected for the insert again by PCR using the α-Factor sequencing primer and 3′ *AOX1* sequencing primer, then colonies having the plasmid were chosen to express Der p 1 in a fermentor system containg methanol. The expressed protein was

purified using His Tag purification protocol and analysed by SDS-polyacrylamide gel electrophoresis.

3.2.7 Expression of recombinant Der p 1 in *Pichia pastoris* cells:

Single colonies of *Pichia* Cells having Der p 1 insert in them were streaked on YPD media and incubated for 2 days at 30°C. A single colony was inoculated in 5ml BMGY media in 50ml tube, grown at 30°C in a shaking incubator at 250 RPM until the culture reached an OD of 2-6 at 600nm. Cells were then harvested at 3000 g for 5 mins at RT, supernatant was removed and cell pellet was resuspended in BMMY media to an OD600 of 1.00 and methanol was added every 24hrs to induce expression. At different time points, 1 ml of the expression media was taken for analysis (Before Induction, 5mins, 30mins, 1hr, 2 hrs, 4hrs, 24 hrs, Day2, Day3 and Day4) to determine the optimal time post-induction to harvest. Samples were kept at -80 until analysis.

3.2.8 Trichloroaceticacid (TCA) precipitation:

In order to concentrate the protein samples, they were precipitated by TCA. Supernatant samples were filter sterilized, then one volume of 100% TCA was added to 4 volumes of protein sample. The mixture was incubated at 4°C for 15 mins, centrifuged at 4000g, supernatant was removed and protein

pellet was washed with cold acetone twice. Pellet was dried at 95°C for 10 mins, then prepared for SDS-PAGE analysis.

3.2.9 Ammonium sulphate sample concentration:

This procedure is useful to fractionate a mixture of proteins. Since large proteins tend to precipitate first, small ones will stay in the solution, thus by analyzing various salt fractions we can find the conditions where the protein we are studying precipitates, and this will increase the purity of protein of interest. A saturated solution of 4.00 M ammonium sulphate was prepared and filter sterilized. Ammonium sulphate was slowly added to cold protein solution while gently mixing until 10% saturation is reached. Samples were incubated for 20 mins on ice with occasional mixing, then the samples were centrifuged and the pellet was collected. More ammonium sulphate was added to the supernatant to reach 20%, 30% and 40% saturation and the pellet was collected from each fraction. Those fractions from each protein preparation were resuspended in suitable buffer and dialyzed and stored for further purifications.

3.2.10 SDS polyacrylamige gel electrophoresis SDS-PAGE:

Cell supernatants were analyzed by electrophoresis using precast 12% PA gels from Invitrogen, tris glycine running buffer (TGS) was prepared and protein samples were run under

reducing conditions for 1hr 30mins at 125V. Protein concentrations on loading were in the range of 5µg to 10 µg.

3.2.11 Coomassie staining analysis:

Gels were washed with deionised H_2O and stained with Coomassie brilliant blue Imperial ready to go stain from Invitrogen for 1hr, they were then destained overnight with H_2O according to the manufacturer protocol.

3.2.12 Silver staining analysis:

Silver staining is a more sensitive tool for protein visualization providing a detection level down to 0.25 ng of protein. The protein detection depends on silver ions binding to amino acid chains of proteins followed by the reduction of these ions giving a metallic silver stain on the gel. Gels were stained using the Dodeca silver staining kit from Biorad according to the manufacturer's protocol.

3.2.13 Western blot analysis:

After gel electrophoresis, nitrocellulose membranes (Amersham, UK) were cut, soaked in tris glycine blotting buffer, gel and membrane were assembled in an Invitrogen transblot apparatus and soaked with blotting buffer according to the manufacturer's protocol. The gels were transferred for 1 hr at 30V. When finished they were blocked overnight with 5% BSA in TBS Tween at 4°C, then incubated for 1 hr with anti-Der p 1 (5H8)

antibody, washed and incubated with anti-mouse secondary antibody conjugated with alkaline phosphatase (AP) and then detected with AP chromogen (NBT-BCIP® solution from Sigma) for 1 hr. After that the reaction was stopped by washing with ultra pure H_2O.

3.2.14 Mutation of Der p 1 glycosylation sites:

The Pro-Der p 1 sequence has 4 possible mutation sites (N16, N82, N132 and N195) (Figs 3-1 & 3-2). Using Stratagene QuikChange® Primer Design program (http://www.stratagene .com/sdmdesigner/default.aspx), primers were designed to change AAC (ASP) Asparagine to CAA (Gln/Q) or Glutamine CAG (Gln/Q) Glutamine in all four mutation sites (Table 3-3).

Table 3-3: Primers used in producing site directed mutations in the glycosylation sites of Wild Type Der p 1 Amplicon.

N16	**Forward N16** **5'-ttttgaagaatacaaaaaagccttccagaaaagttatgctaccttcgaagatg-3'** **Reverse N16** **3'-aaaacttcttatgttttttcggaaggtcttttcaatacgatggaagcttctac-5**
N82	**Forward N82** **5'-caaaactcaattcgatttgaatgctgaaactcaggcctgcagtatcaatg-3'** **Reverse N82** **3'-gttttgagttaagctaaacttacgactttgagtccggacgtcatagttac-5'**
N132	**Forward N132** **5'-atcagcttatttggcttaccgtcagcaatcattggatcttgctgaac-3'** **Reverse N132** **3'-tagtcgaataaaccgaatggcagtcgttagtaacctagaacgacttg-5'**
N195	**Forward N195** **5'-cacaacgtttcggtatctcacagtattgccaaatttacccacc-3'** **Reverse N195** **3'-gtgttgcaaagccatagagtgtcataacggtttaaatgggtgg-5'**

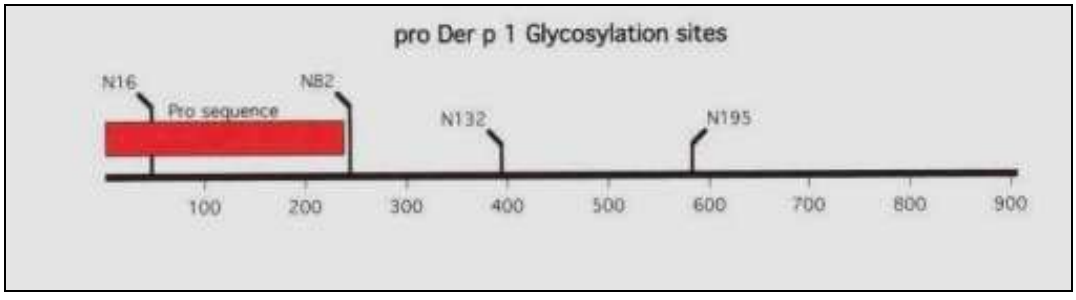


Fig 3-1: diagram of glycosylation sites in pro Der p 1 DNA sequence.

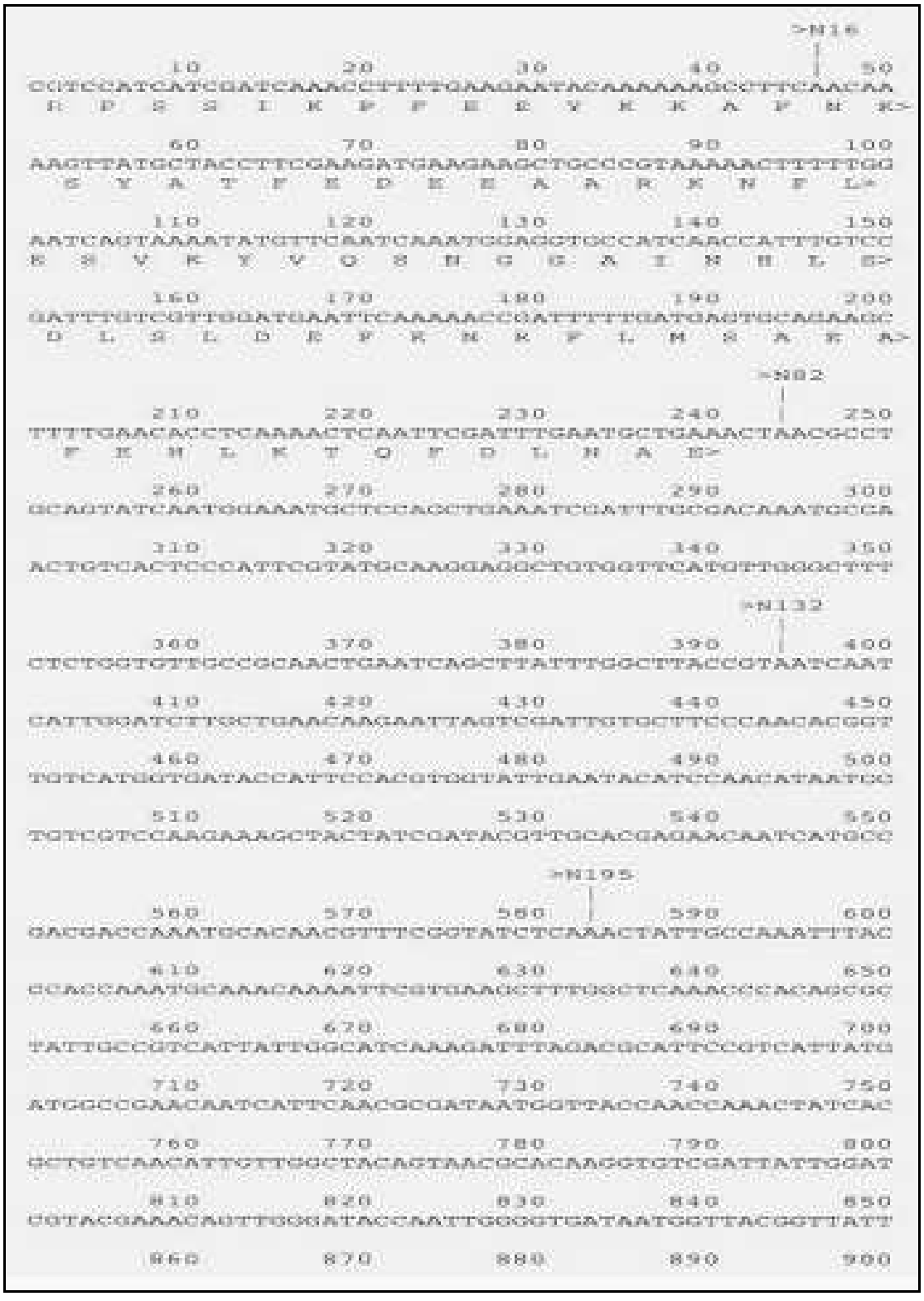


Fig 3-2: Glycosylation sites in pro Der p 1 DNA sequence.

3.2.15 The Quick change site directed mutagenesis method:

Using Stratagene QuikChange® kit DNA plasmids containing N16, N82, N132 and N195, mutants were denatured, the mutagenic primers annealed and extended with *PfuUltra* DNA polymerase (Fig 3-3), the parental plasmid were digested with DpnI and all plasmids were transformed to TOP 10 *E. coli* competent cells, separately. Some colonies were selected for sequencing to detection of the desired mutation. Plasmids were then propagated and purified from *E. coli* cells using PureLink Quick plasmid Miniprep Kit according to manufacturer protocol.

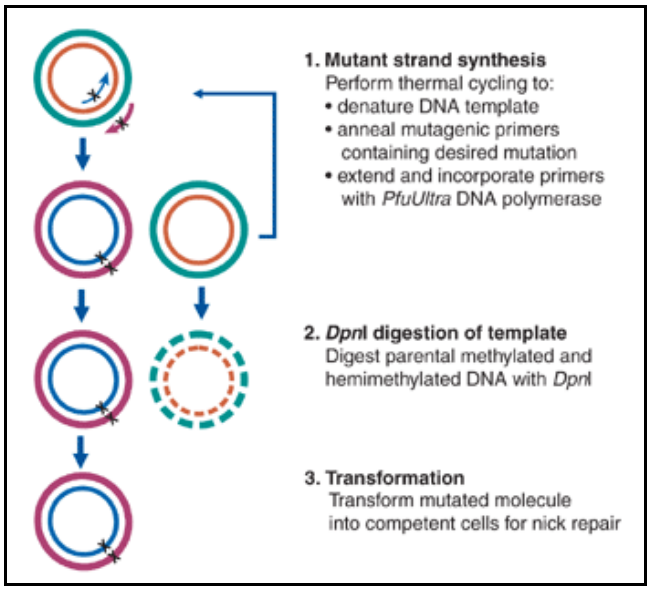


Fig 3-3: The Quick change site directed mutagenesis method.

The PCR reaction conditions were as follow:

5 µl of 10× reaction buffer

2 µl (10 ng) of pWhitescript 4.5-kb control plasmid (5 ng/µl)

1.25 µl (125 ng) of oligonucleotide control primer #1

1.25 µl (125 ng) of oligonucleotide control primer #2

1 µl of dNTP mix

38.5 µl ddH2O (to bring the final reaction volume to 50 µl)

Then add

1 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl)

Segment Cycles Temperature Time

1X

95°C 30 seconds

25x

95°C 30 seconds

55°C 1 minute

68°C 13mins

1 µl of the *Dpn* I (10 U/µl) restriction enzyme was added directly to each amplification reaction and digest at 37° C for 1 hr. Cells were transformed to *E.coli* TOP 10 Competent cells.

3.2.16 Sequence analysis and alignments:

Sequencing reactions using Big Dye v1.1 were done according to the following conditions: denaturing at 96°C for 30 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 mins, then a final extension at 28°C for 1min.

Then samples were precipitated by ethanol precipitation and sequenced using the ABI 3130 analyser to confirm that the PCR

products were for the cDNA of pro-Der p 1. Sequence analysis to confirm mutations in the glycosylation sites for Der p 1 was also done. Comparison alignment for the mutants to the wild type Der p 1 was done using the Blast search engine to detect site mutations (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 3-4: primers used to sequence Wild Type Der p 1 and mutants to detect differences.

Primer	Sequence
5´ *AOX1* sequencing primer (5´ *Pichia* primer)	5´-GACTGGTTCCAATTGACAAGC-3´
3´ *AOX1* sequencing primer (3´ *Pichia* primer)	5´-GCAAATGGCATTCTGACATCC-3´
α-Factor sequencing primer	5´-TACTATTGCCAGCATTGCTGC-3´

3.2.17 Transformation of N16, N82, N132 and N195 Der p 1 mutants to TOP 10 *E. coli* competent cells:

TOP 10 *E. coli* chemically competent cells (from Invitrogen) were used to transform N16, N82, N132 and N195 Der p 1 mutants in pPICZ α A vector. Competent cells were kept chilled on ice during transformation, and up to 40.0ng of DNA (Plasmid with Insert) in 10 µl volume was mixed with 80 µl competent cells. For the positive control, 10.0 ng of pPICZ α A was mixed with 80 µl competent cells, while for the negative control no vector was added to the competent cells. All mixtures were immediately

placed on ice for 30 minutes, then they were heat shocked at 42°C for 45 sec, then 900 µl of LB media was added to the mixtures and incubated for 1 hr at 37°C. The reaction was then plated on LB Zeocin plates overnight at 37°C. Colonies that grew overnight were detected by PCR using the α-Factor sequencing primer and 3´ *AOX1* sequencing primer to check for the plasmid. The PCR reaction conditions were as follow: initial denaturation at 94°C for 10 mins, followed by 35 cycles of denaturing at 94°C for 90 mins, annealing at 54°C for 90 mins and extension at 72°C for 2 mins and a final extension at 72°C for 2 mins. Only colonies having the Der p 1 insert were selected, streaked and then grown in LB Zeocin culture overnight at 37°C.

3.2.18 Purification of plasmid from *E.coli* TOP 10 culture:

Purification of N16, N82, N132 and N195 pPICZ α A vector from *E. coli* TOP 10 culture was done using PureLink Quick plasmid Miniprep Kit according to manufacturers' protocol. The concentration of plasmid was measured with the nanodrop ND 1000 spectrophotometer. The purified plasmid was then run on a 2% agarose e-gel to confirm its purification and it was sequenced to ensure that it is the pPICZ α A vector. All plasmids were then sequenced using Big Dye v1.1 sequencing reaction to confirm the correct mutation in the Der p 1 sequence.

3.2.19 Transformation of pro and mature Der p 1 insert in to X33 yeast competent cells:

Competent cells from X33 and GS115 yeast cell culture (provided in the easyComp kit from Invitrogen) were prepared acoording to manufactorer protocol.The plasmid used was linearized using SacI restriction digestion at 37°C for 2 Hours using 5.0 U of Sac I to Digest 5 µg of plasmid DNA. Buffer 1 was added to the reaction provided from New England BioLabs. Then up to 5 µg of the linearized plasmid was transferred to *Pichia pastoris* X33 and GS115 competent cells using the EasyComp transformation kit from invitrogen according to the kit protocol. Positive and negative controls were introduced during transformation reaction. After that *Pichia* cells were plated on YPD Zeocin containg media at 30°C for 4 days and the colonies that were growing were detected for the insert again by PCR using the α-Factor sequencing primer and 3′ *AOX1* sequencing primer, then colonies having the plasmid were chosen to express Der p 1 in a fermentor system containg methanol.

3.2.20 Expression of different glycoforms in *Pichia pastoris* X33 and GS115 cells:

Using a single colony, 5 ml BMGY (Buffered Glycerol-complex Medium) media were inoculated in a 50 ml tube, and grown at 28–30°C in a shaking incubator (250–300 RPM) until culture

reaches an OD600 = 2 (the OD recommended by Invitrogen is 2-6). Such cells will be in early log-phase growth. Cells were then harvested by centrifuging at 1,500–3,000 g for 5 minutes at room temperature. Supernatant was then decanted and cell pellet was resuspended to an OD600 of 1.0 in BMMY (Buffered Methanol-complex Medium) to induce expression.

At each of the time points (before Induction, 5mins, 30mins, 1hr, 2 hrs, 4hrs, 24 hrs, Day2, Day3 and Day4), 1 ml of the expression culture was transferred to a 1.5 ml microcentrifuge tube. These samples were used to analyze expression levels and determine the optimal time post-induction to harvest.

Supernatants were then transferred to a separate tube and stored at –80°C until ready to assay. Analysis by silver staining and western blot was made for Der p1 expression.

3.3 Results:

3.3.1 PCR amplification for the cDNA of Mature and pro Der p 1:

Phenol/chloroform extraction was done for both libraries to purify the cDNA. In λ phage 10 the concentration for cDNA was 4101.3 ng/µl and in λ phage 11 the concentration for cDNA was 3781.4 ng/µl.

Mature and pro Der p 1 sequences were amplified by PCR using the primers listed in table 1. They were run on 0.8% agarose e-gel for detection (Fig 3-4).

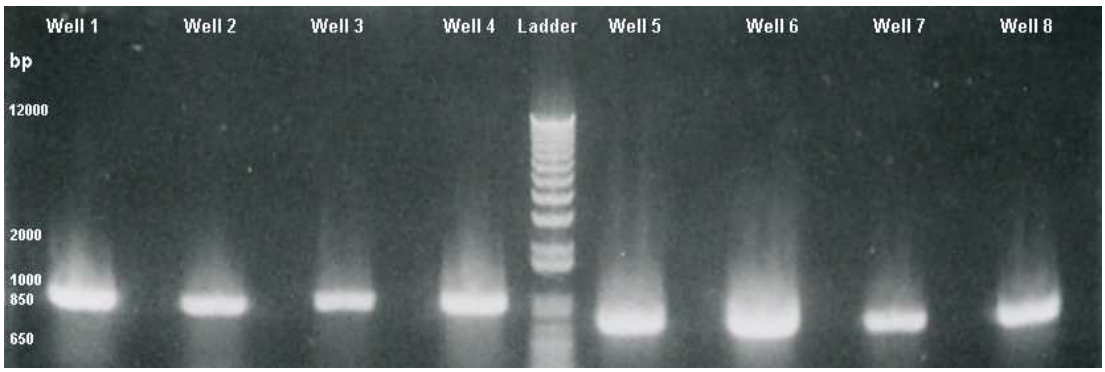


Fig 3-4: amplification of pro and mature Der p 1 from λ phage 11 library. Ladder: 1 Kb plus DNA ladder, well 1-2: amplified pro Der p 1 using primers (4n+p1), well 3-4: amplified pro Der p 1 using primers (4s+p1), well 5-6: amplified mature Der p 1 using primers (4n+p3), and well 7-8: amplified mature Der p 1 using primers (4s+p3).

3.3.2 Purification of Der p 1 PCR product:

The PCR products for the pro and mature Der p 1 were purified using QIAquick PCR purification kit according to manufacturer protocol, and its concentrations were measured using the nanodrop ND 1000 spectrophotometer (Table 3-5), then the product was run on agarose e-gel for detection (Fig 3-5).

Table 3-5: Der p 1 purified PCR product concentrations**.**

Purified PCR Sample	Concentration ng/µl
PCR Sample 1 (pro Der p 1)	15 ng/µl
PCR Sample 2 (pro Der p 1)	37.7 ng/µl
PCR Sample 3 (pro Der p 1)	38.1 ng/µl
PCR Sample 4 (pro Der p 1)	33.3 ng/µl
PCR Sample 5 (mature Der p 1)	49.3 ng/µl
PCR Sample 6(mature Der p 1)	38.9 ng/µl
PCR sample 7(mature Der p 1)	27.2 ng/µl
PCR sample 8(mature Der p 1)	29.0 ng/µl

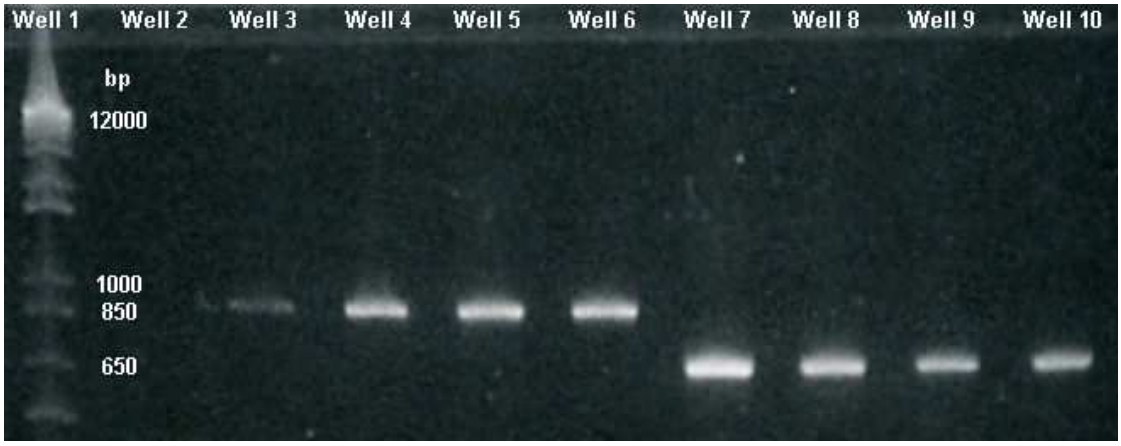


Fig 3-5: PCR product run on gel after purification. Well 1: 1 Kb plus DNA ladder. Well 3- 6: Purified PCR samples for pro Der p 1. Well 7-10: Purified PCR samples for mature Der p 1.

ABI 3130 analyser was used to confirm that the PCR products are for the cDNA of mature and pro Der p 1. Comparison alignment for the amplified PCR product of the mature and pro Der p 1 with gene

bank data for the complete Der p 1 allergen shows compatibility (Figs 3-6, 3-7).

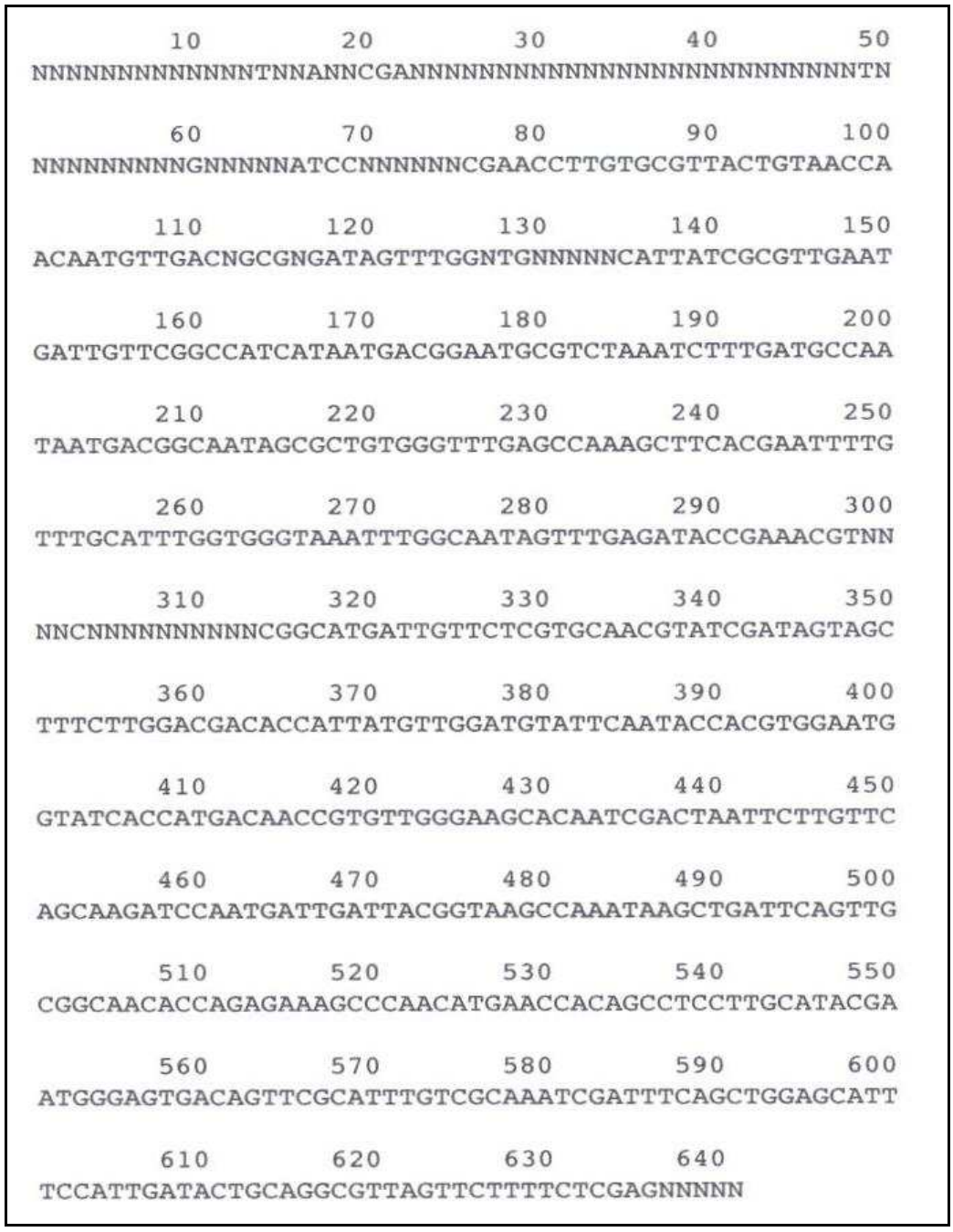
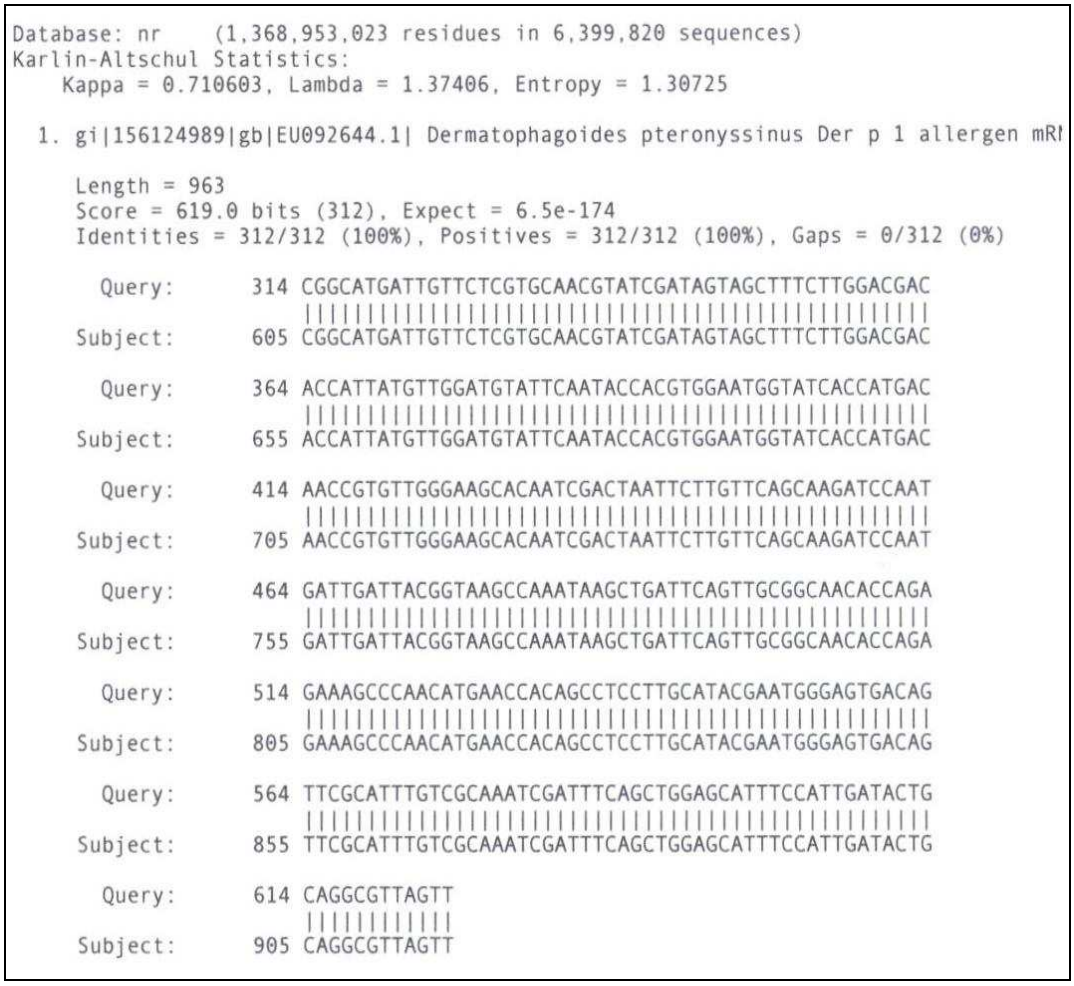


Fig 3-6: Sequencing results for the mature Der p 1 PCR product.



```
Database: nr    (1,368,953,023 residues in 6,399,820 sequences)
Karlin-Altschul Statistics:
    Kappa = 0.710603, Lambda = 1.37406, Entropy = 1.30725

 1. gi|156124989|gb|EU092644.1| Dermatophagoides pteronyssinus Der p 1 allergen mRI

    Length = 963
    Score = 619.0 bits (312), Expect = 6.5e-174
    Identities = 312/312 (100%), Positives = 312/312 (100%), Gaps = 0/312 (0%)

      Query:      314 CGGCATGATTGTTCTCGTGCAACGTATCGATAGTAGCTTTCTTGGACGAC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    Subject:      605 CGGCATGATTGTTCTCGTGCAACGTATCGATAGTAGCTTTCTTGGACGAC

      Query:      364 ACCATTATGTTGGATGTATTCAATACCACGTGGAATGGTATCACCATGAC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    Subject:      655 ACCATTATGTTGGATGTATTCAATACCACGTGGAATGGTATCACCATGAC

      Query:      414 AACCGTGTTGGGAAGCACAATCGACTAATTCTTGTTCAGCAAGATCCAAT
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    Subject:      705 AACCGTGTTGGGAAGCACAATCGACTAATTCTTGTTCAGCAAGATCCAAT

      Query:      464 GATTGATTACGGTAAGCCAAATAAGCTGATTCAGTTGCGGCAACACCAGA
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    Subject:      755 GATTGATTACGGTAAGCCAAATAAGCTGATTCAGTTGCGGCAACACCAGA

      Query:      514 GAAAGCCCAACATGAACCACAGCCTCCTTGCATACGAATGGGAGTGACAG
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    Subject:      805 GAAAGCCCAACATGAACCACAGCCTCCTTGCATACGAATGGGAGTGACAG

      Query:      564 TTCGCATTTGTCGCAAATCGATTTCAGCTGGAGCATTTCCATTGATACTG
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    Subject:      855 TTCGCATTTGTCGCAAATCGATTTCAGCTGGAGCATTTCCATTGATACTG

      Query:      614 CAGGCGTTAGTT
                      ||||||||||||
    Subject:      905 CAGGCGTTAGTT
```

Fig 3-7: Comparison alignment for the amplified PCR product of the mature Der p 1 with gene bank data for the complete Der p 1 allergen shows compatibility.

3.3.3 Ligation of pro and mature Der p 1 PCR insert in pPICZ α A vector:

The purified PCR product of pro and mature Der p 1 was ligated to pPICZ α A Vector (Fig 3-8). First, restriction digestion using KpnI and Xho I was done for both the PCR product and pPICZ α A vector, then the ligation reaction was carried out for Der p 1 and pPICZ α A vector using Ready-To-Go T4 DNA ligase kit according to manufacturer protocol.

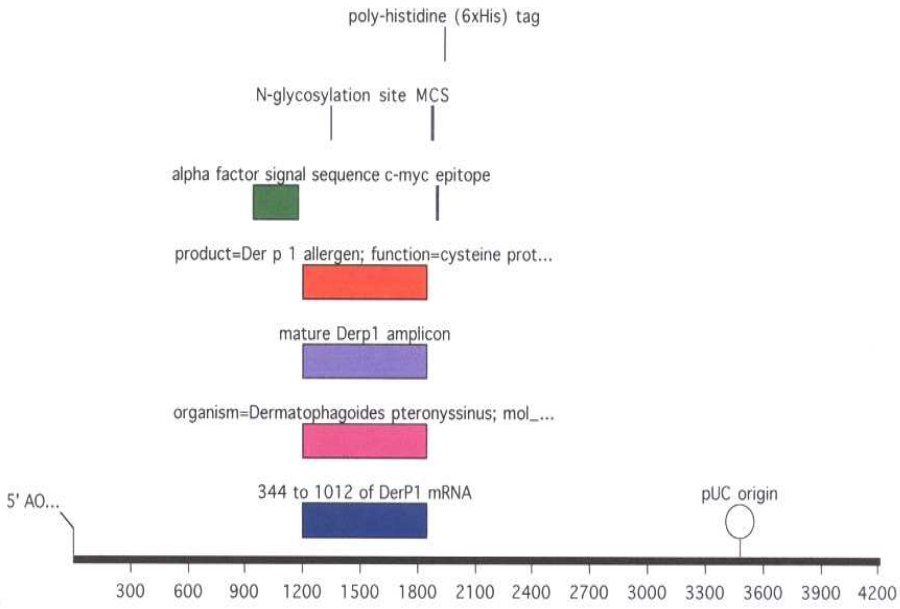


Fig 3-8: pPICZ α A vector with mature Der p 1 insert in. (Diagram drawn by MacVector program Ver.7)

3.3.4 pPICZ α A Vector (with pro and mature Der p 1 insert) transformation into *E. coli*:

By using chemically competent TOP 10 *E. coli* cells the plasmid was transferred using chemical transformation method. Positive

and negative controls were used during transformation (Fig 3-9). In order to detect if pro and mature Der p 1 insert is ligated to the plasmid in the transformed colonies, a PCR reaction was made using primers close to Der p 1 Insert location in the plasmid to amplify a piece of the plasmid with Der p 1 insert in it (Fig 3-10).

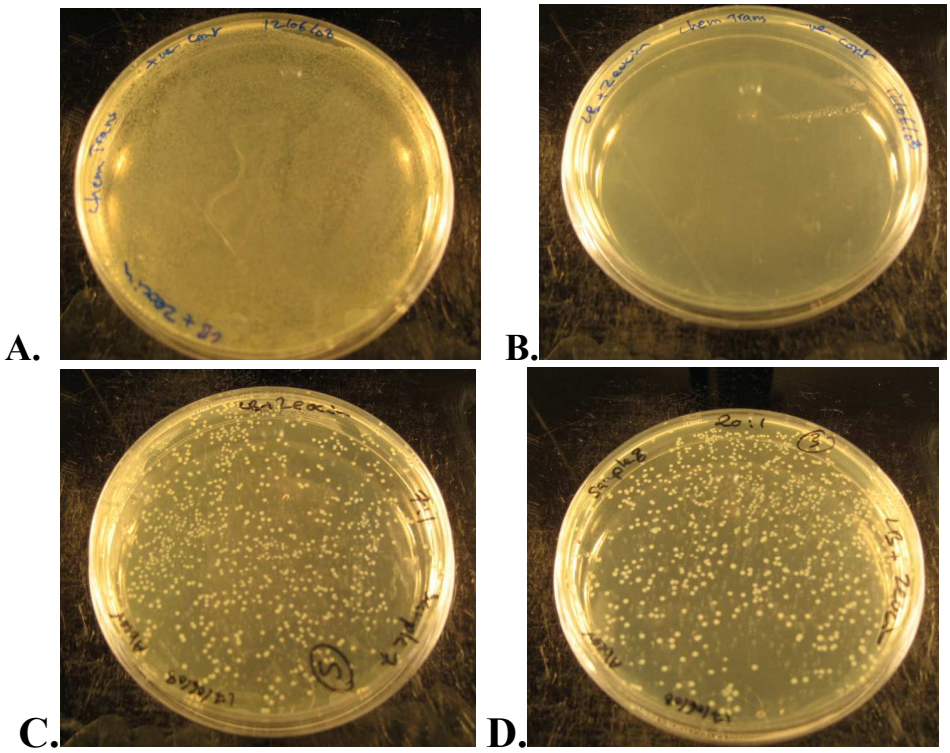


Fig 3-9: Chemical transformation of pPICZα A vector with Der p 1 insert in TOP 10 *E.coli*. **A.** positive control for pPICZα A vector transformation with no insert. The plate shows many colonies **B**. negative control (no plasmid), **C** and **D** Chemical transformation of pPICZα A with Der p 1 insert in TOP 10 *E.coli*.

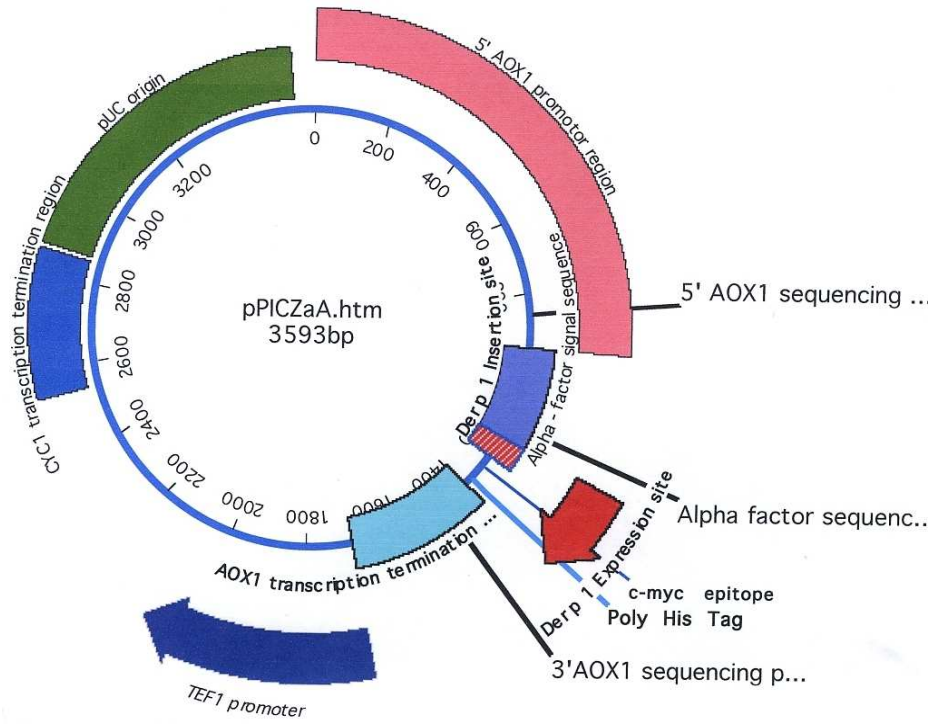


Fig 3-10: Map for primers location in pPICZ α A vector. (Diagram drawn by MacVector program Ver.7)

3.3.5 Detection of Der p 1 insert in transformed TOP 10 *E. coli* colonies:

In order to detect if pro and mature Der p 1 insert is ligated to the plasmid in the transformed colonies, before tranforming this plasmid to yeast cells, a PCR reaction was made using primers close to Der p 1 insert location in the plasmid to amplify a piece of the plasmid with Der p 1 insert in it. By using the α-Factor sequencing primer and the 3´ *AOX1* sequencing primer (Table 3-6), a PCR fragment of 918 bp should appear on the gel if the Der p 1 insert is in, if it is not in, the PCR fragment should be 266 bp (Fig 3-11).

Table 3-6: primers used to detect Der p 1 insert in pPICZ α A vector

Primer	Sequence
5´ *AOX1* sequencing primer (5´ *Pichia* primer)	5´-GACTGGTTCCAATTGACAAGC-3´
3´ *AOX1* sequencing primer (3´ *Pichia* primer)	5´-GCAAATGGCATTCTGACATCC-3´
α-Factor sequencing primer	5´-TACTATTGCCAGCATTGCTGC-3´

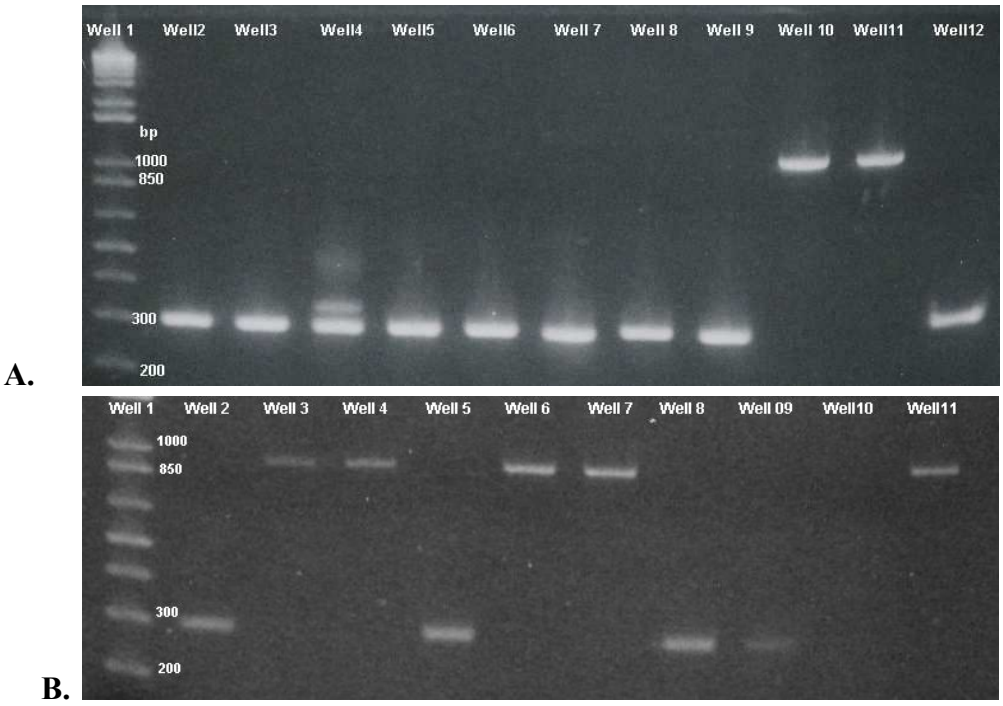


Fig 3-11: PCR amplification of pPICZ α A plasmid from *E. coli* TOP 10 transformed colonies. Figure A: Well 1 : 1 Kb plus DNA ladder. Well 2-9, 12: PCR amplification for colonies having pPICZα a plasmid with no pro Der p 1 insert (266 bp) . Well: 10-11: PCR amplification for colonies having pPICZα a plasmid with pro Der p 1 Insert (up to 1000 bp). Figure B: Well 1: 1 Kb plus DNA ladder. Well 2, 5, 8, 9: PCR amplification for colonies having pPICZα a plasmid with no mature Der p 1 insert (266 bp). Well 3, 4, 6, 7, 11: PCR amplification for colonies having pPICZα a plasmid with mature Der p 1 Insert (918 bp).

The presence of mature (Wells 3, 4, 6, 7 and 11 in figure 3-11 B) and pro Der p 1 (Well 10 and 11 in Figure 3-11) was confirmed by sequencing those PCR fragments using the ABI

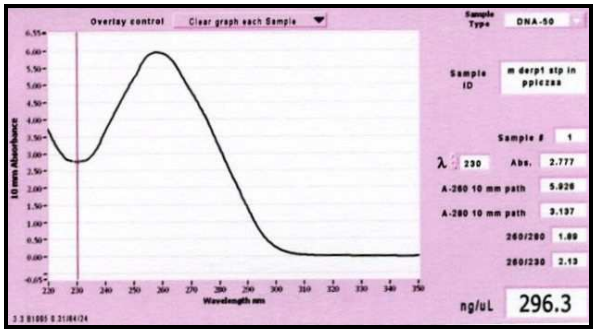
3130 analyser and comparing sequences with plasmid and Der p 1 sequenced data.

Only the colonies having the mature and proDer p 1 insert were streaked on LB media Zeocin selective plates, then pure colonies were cultured overnight in LB zeocin selective media in a shaking incubator at 37°C.

3.3.6 Purification of plasmid from *E.coli* TOP 10 culture:

Purification of pPICZ α A vector from *E. coli* TOP 10 culture was done using PureLink Quick plasmid Miniprep Kit according to manufacturer protocol. The concentration of plasmid was measured with the nanodrop ND 1000 spectrophotometer. The purified plasmid was then run on 2% agarose e-gel to confirm its purification and it was sequenced to assure that it is pPICZ α A vector (Fig 3-12).

A.



B.

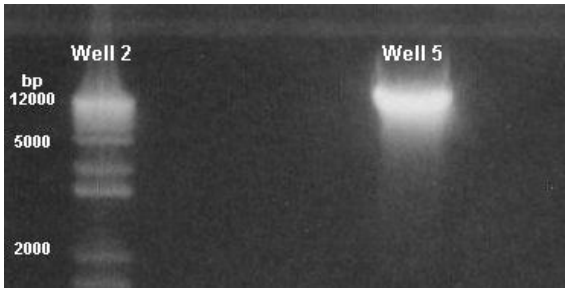


Fig 3-12: A. Concentration of purified pPICZ α A vector from *E. coli* culture. B. Well 2: 1 Kb plus DNA ladder. Well 5: purified pPICZ α A vector from *E. coli*

3.3.7 Transformation to X33 yeast competent cells:

By using chemically competent X33 yeast cells, the purified linear plasmid was chemically transformed to yeast cells and plated on YPD Zeocin agar plates. The plates were incubated at 30ºC for 2 days. The cells that grew were detected for the pPICZ α A vector using PCR. Colonies detected by PCR having the plasmid in were chosen to express Der p 1 in methanol fermentation system (Fig 3-13).

A.  B. 

C.  D. 

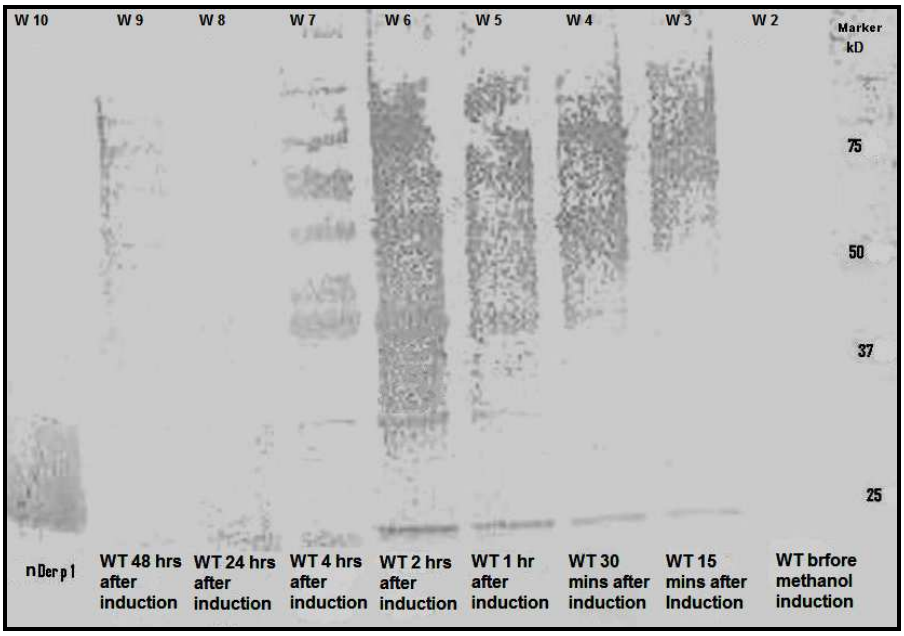
Fig 3-13: Chemical transformation of pPICZαA with Der p 1 insert to X33 chemically competent cells. A. positive control for pure pPICZα A vector transformation with no insert. B. negative control C and D Chemical transformation of pPICZα A with Der p 1 insert in X33 yeast cells.

3.3.8 Expression of rDer p 1 in *Pichia pastoris* X33 and GS115 yeast cells:

Der p 1 was expressed in yeast cells and detected by western blot against anti-Der p 1 (5H8). A band that reacted with 5H8 could be detected at 25 kDa in the culture after 4 days of induction. It was also noticed that a diffuse band of a MW between 50 and 100 kD appeared after 5 mins of methanol

induction then dissociated after day 1 of induction. In the same culture in day 1,there was also a major band at 40 kD after 4 hrs of induction (Fig 3-14 A)and another band at 25 kD also reacted with 5H8 suggesting expression of Der p 1 might be of different glycosylation forms by the yeast cells (Fig 3-14 A) or that the culture proteases are affecting the expression. These results were inconsistent and could not be repeated. In Fig 3-14 (B) another expression of a different colony can be detected.

A.



B.

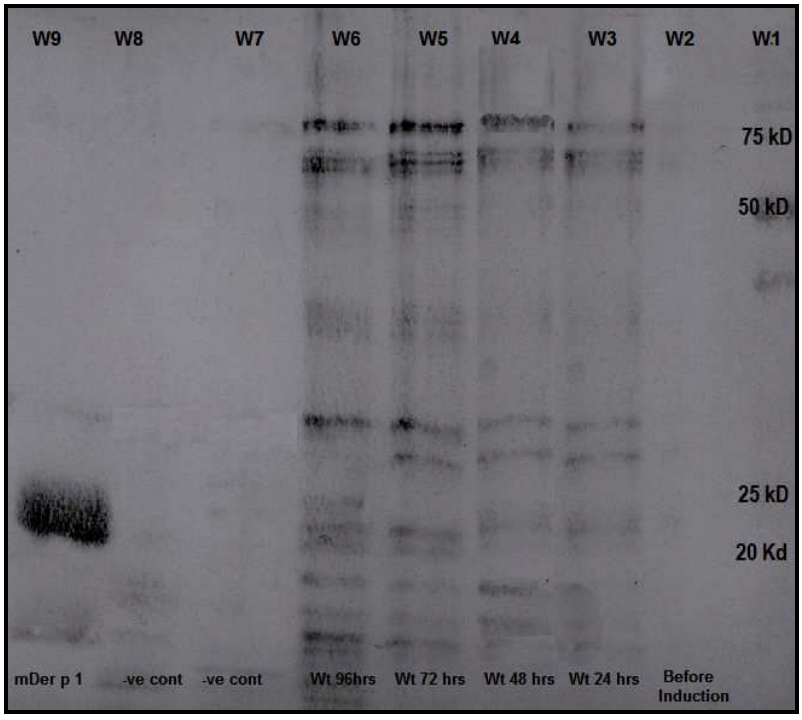


Fig 3-14: Time point expression of WT Der p 1 (pro Der p 1) and mature Der p 1 in *Pichia pastoris* cells before and after methanol induction to induce expression. This is a western blot against anti Der p 1 5H8 for two separate experiments of expression (A and B).

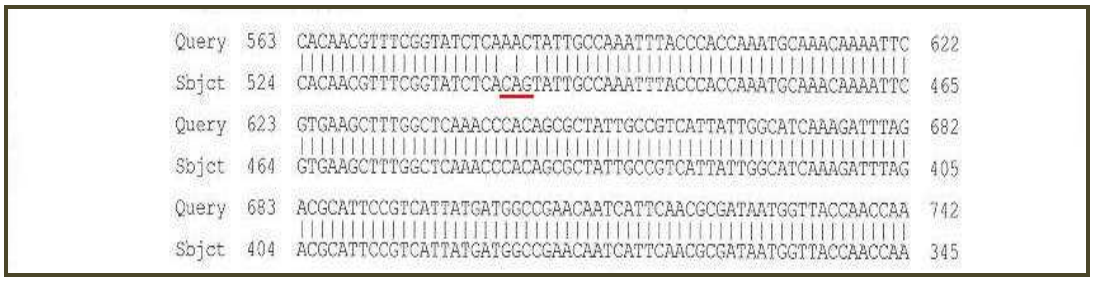
3.3.9 Mutagenesis of the glycosylation sites in Der p 1 amplicon:

Quick change site directed mutagenesis method was used to construct point mutations in N16, N82, N132 and N195 Der p 1 glycosylation sites. The new plasmids were transferred to TOP 10 cells and the colonies that grow at 37°C were streaked, single colonies were chosen from the plate, cultured in LB media ON at 37°C and the plasmids were purified from the culture ran on a gel then sequenced and aligned to the wild type unchanged plasmid to confirm mutagenesis. It was found that all four Asparagine residues in four plasmids were changed to Glutamine by changing ACC to CAG (Figs 3-15, 3-16, 3-17, 3-18).

N16

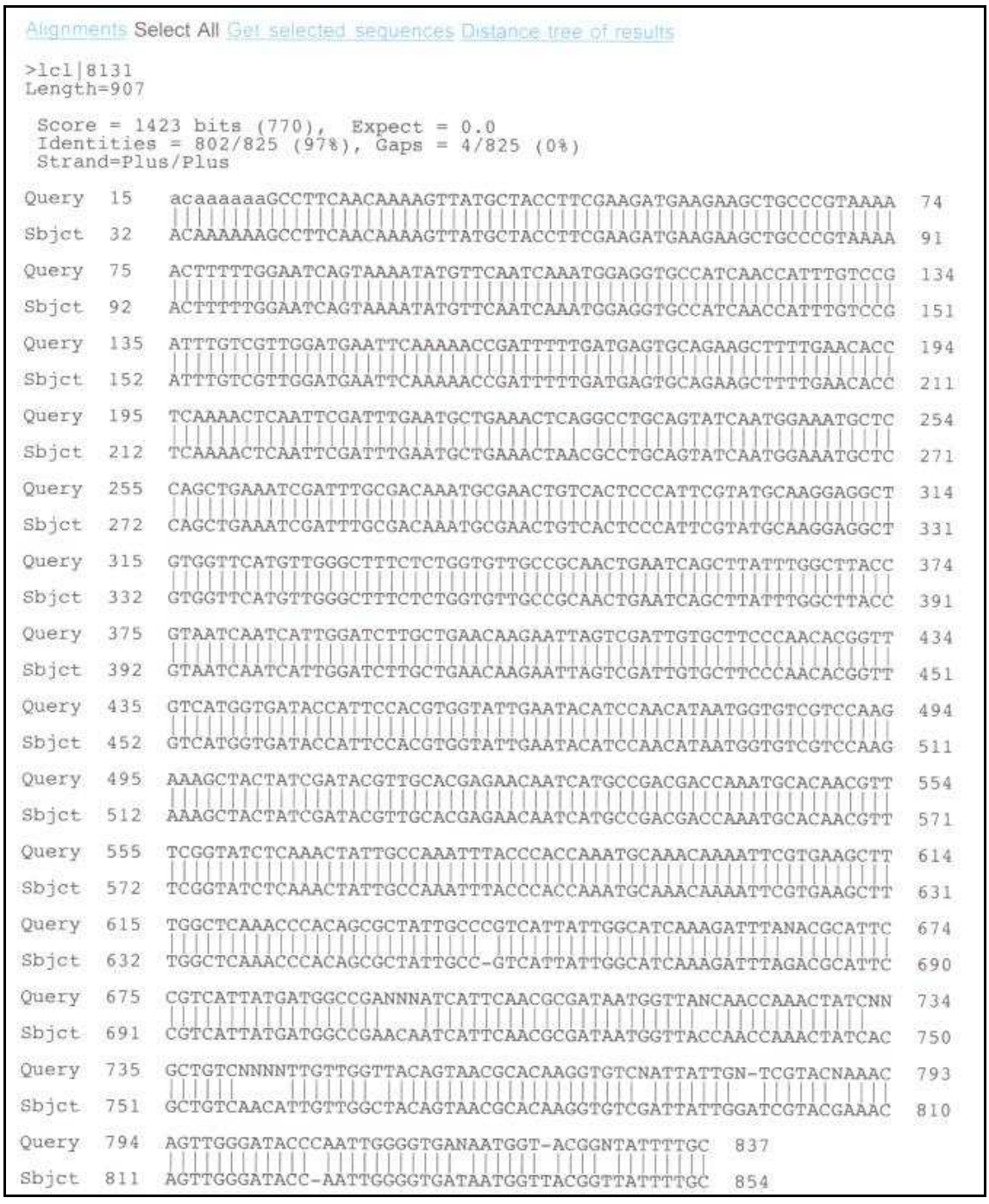
N82

N132



N195

Fig 3-15: Sequence alignment comparisons of Site mutations (N16, N82, N132, N195) in Der p 1 insert compared to wild type.



Alignments Select All Get selected sequences Distance tree of results

>lcl|8131
Length=907

Score = 1423 bits (770), Expect = 0.0
Identities = 802/825 (97%), Gaps = 4/825 (0%)
Strand=Plus/Plus

```
Query  15   acaaaaaaGCCTTCAACAAAAGTTATGCTACCTTCGAAGATGAAGAAGCTGCCCGTAAAA  74
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32   ACAAAAAAGCCTTCAACAAAAGTTATGCTACCTTCGAAGATGAAGAAGCTGCCCGTAAAA  91

Query  75   ACTTTTTGGAATCAGTAAAATATGTTCAATCAAATGGAGGTGCCATCAACCATTTGTCCG  134
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92   ACTTTTTGGAATCAGTAAAATATGTTCAATCAAATGGAGGTGCCATCAACCATTTGTCCG  151

Query  135  ATTTGTCGTTGGATGAATTCAAAAACCGATTTTTGATGAGTGCAGAAGCTTTTGAACACC  194
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  152  ATTTGTCGTTGGATGAATTCAAAAACCGATTTTTGATGAGTGCAGAAGCTTTTGAACACC  211

Query  195  TCAAAACTCAATTCGATTTGAATGCTGAAACTCAGGCCTGCAGTATCAATGGAAATGCTC  254
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  TCAAAACTCAATTCGATTTGAATGCTGAAACTAACGCCTGCAGTATCAATGGAAATGCTC  271

Query  255  CAGCTGAAATCGATTTGCGACAAATGCGAACTGTCACTCCCATTCGTATGCAAGGAGGCT  314
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  CAGCTGAAATCGATTTGCGACAAATGCGAACTGTCACTCCCATTCGTATGCAAGGAGGCT  331

Query  315  GTGGTTCATGTTGGGCTTTCTCTGGTGTTGCCGCAACTGAATCAGCTTATTTGGCTTACC  374
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  GTGGTTCATGTTGGGCTTTCTCTGGTGTTGCCGCAACTGAATCAGCTTATTTGGCTTACC  391

Query  375  GTAATCAATCATTGGATCTTGCTGAACAAGAATTAGTCGATTGTGCTTCCCAACACGGTT  434
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Sbjct  392  GTAATCAATCATTGGATCTTGCTGAACAAGAATTAGTCGATTGTGCTTCCCAACACGGTT  451

Query  435  GTCATGGTGATACCATTCCACGTGGTATTGAATACATCCAACATAATGGTGTCGTCCAAG  494
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Sbjct  452  GTCATGGTGATACCATTCCACGTGGTATTGAATACATCCAACATAATGGTGTCGTCCAAG  511

Query  495  AAAGCTACTATCGATACGTTGCACGAGAACAATCATGCCGACGACCAAATGCACAACGTT  554
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  555  TCGGTATCTCAAACTATTGCCAAATTTACCCACCAAATGCAAACAAAATTCGTGAAGCTT  614
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  572  TCGGTATCTCAAACTATTGCCAAATTTACCCACCAAATGCAAACAAAATTCGTGAAGCTT  631

Query  615  TGGCTCAAACCCACAGCGCTATTGCCCGTCATTATTGGCATCAAAGATTTANACGCATTC  674
            ||||||||||||||||||||||| |||||||||||||||||||||||||| ||||||||||
Sbjct  632  TGGCTCAAACCCACAGCGCTATTGCC-GTCATTATTGGCATCAAAGATTTAGACGCATTC  690

Query  675  CGTCATTATGATGGCCGANNNATCATTCAACGCGATAATGGTTANCAACCAAACTATCNN  734
            |||||||||||||||| |||| |||||||||||||||||||| ||||||||||||||||||
Sbjct  691  CGTCATTATGATGGCCGAACAATCATTCAACGCGATAATGGTTACCAACCAAACTATCAC  750

Query  735  GCTGTCNNNNTTGTTGGTTACAGTAACGCACAAGGTGTCNATTATTGN-TCGTACNAAAC  793
            |||||| |||||||||||||||||||||||||||||||| |||||| | ||||| |||||
Sbjct  751  GCTGTCAACATTGTTGGCTACAGTAACGCACAAGGTGTCGATTATTGGATCGTACGAAAC  810

Query  794  AGTTGGGATACCCAATTGGGGTGANAATGGT-ACGGNTATTTTGC  837
            ||||||||||| |||||||||| |||||||| |||| |||||||
Sbjct  811  AGTTGGGATACC-AATTGGGGTGATAATGGTTACGGTTATTTTGC  854
```

Fig 3-16: The whole sequence of N82 Der p 1 compared to the Wild Type Der p 1.

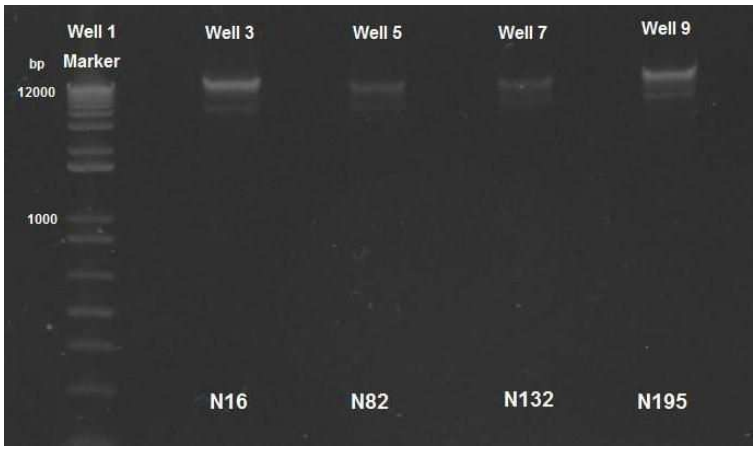
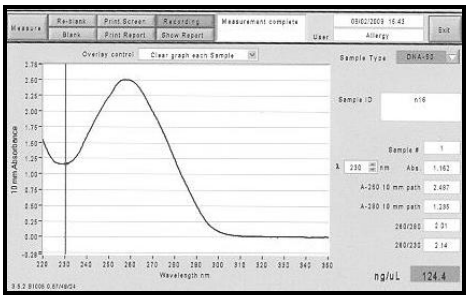
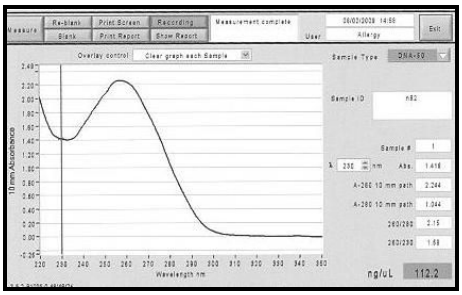


Fig 3-17: Purified fractions of mutant Der p 1 plasmids (N16, N82, N132 and N195) ran on 2% e. gels to detect pure plasmids.

A.  B. 

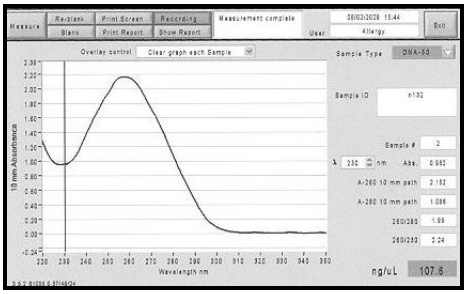
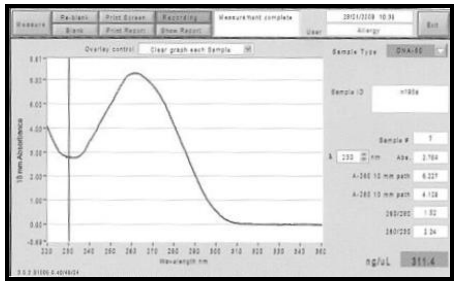
C.  D. 

Fig 3-18: Purified fractions of mutant Der p 1 plasmids measured by the nanodrop. A. N16; B. N82; C. N132; D. N195.

3.3.10 Plasmids N16, N82, N132 and N195 transformation to X33 competent *Pichia* cells:

Plasmids having Der p 1 insert with mutants in each of its glycosylation site were concentrated and transformed to X33 competent *Pichia.* Colonies were grown for 4 days at 30° C; positive and negative controls were introduced to monitor transfer. For the positive control the uncut pPiczαA was used and no vector was used with the negative control (Fig 3-19).

A.  B.  C. 

D.  E.  F. 

Fig 3-19: Chemical transformation of pPICZαA with Der p 1 inserts [(A) Wild Type, (B) Positive control the uncut pPiczαA with no Der p 1 insert and (C-F) mutants] to X33 chemically competent cells.

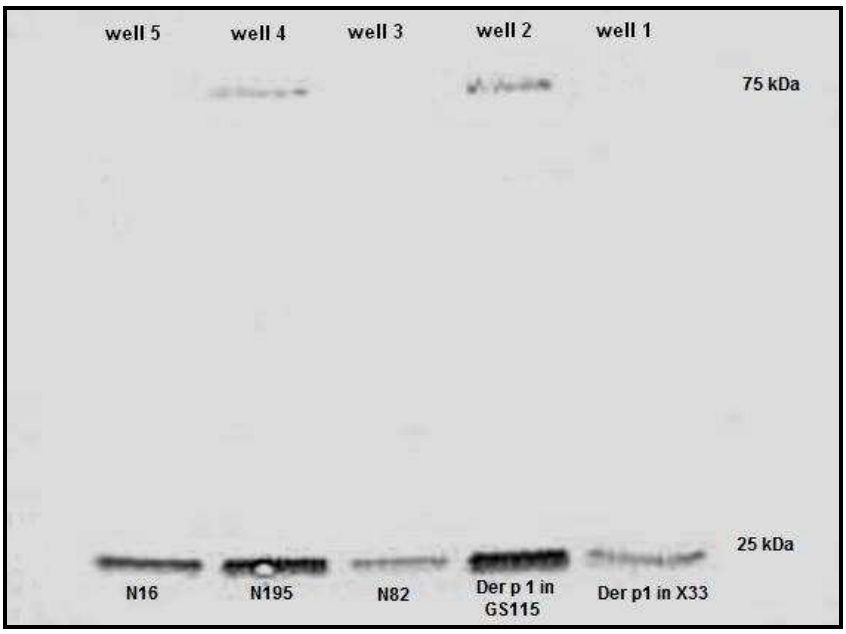
3.3.11 Expression of different glycoforms in *Pichia* cells:

All four Der p 1 mutants were expressed in yeast cells. Cells were cultured in BMGY media as a starter culture, then to induce expression they were transferred to BMMY media with continual

induction by methanol for four days at 30°C. Cultures were then harvested and prepared for detection of expression. Both 5H8 and 2C7 anti-Der p 1 antibodies were used to detect expression (Fig 3-20).

It was noticed that the different Der p 1 mutants expressed at 25 kDa indicating aglycosylation.

A.



B.

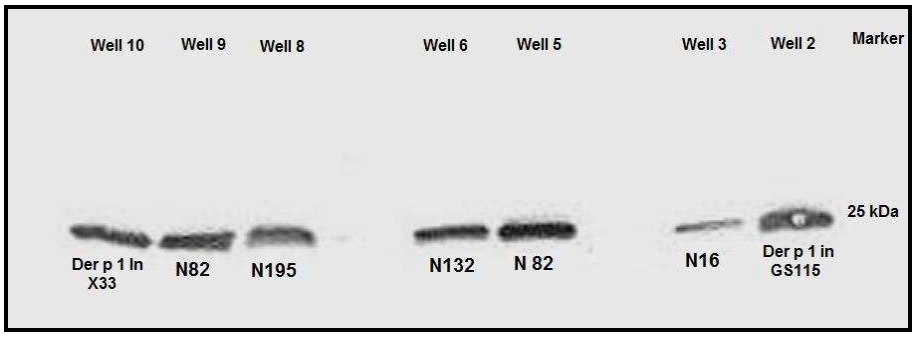


Fig 3-20: Immunoblot against anti-Der p1 (5H8) showing the expression of Der p 1 glycoforms in X33 and GS115 yeast cells (A and B).

3.4 Discussion:

Der p 1 is a cysteine protease allergen that is recognised as being one of the most potent indoor allergens (Altmann, 2007, Adnan et al., 1996, Charbonnier et al., 2003, Deb et al., 2007, Ghaemmaghami et al., 2002, Shakib et al., 1998).

In this work, the Der p 1 cDNA PCR product was successfully amplified from a phage library, sequenced, inserted into a vector, transformed to TOP 10 cells for amplification and concentration. The vector was then purified, concentrated, transformed to yeast X33 competent cells and expressed. Mutants of possible glycosylation sites in the proDer p 1 amplicon were produced using quick-change site directed mutagenesis kit. The four plasmids having those mutants were then sequenced, transformed to TOP 10 cells then purified, concentrated, transformed to yeast X33 competent cells and expressed.

Der p1 and four deglycosylated mutants (N16, N82, N132, N195) were successfully expressed in Yeast as shown by their reaction to anti-Der p 1 5H8. However, the expressed protein glycosylation profile in yeast cells seem to be controlled by multiple factors. Methanol induction, carbon source and growth conditions of the cells are major factors that control the degree of glycosylation of expressed proteins in *Pichia*. A high molecular

weight form of Der p 1, indicating hyperglycosylation, was detectable on the first day of expression after inducing the cells with methanol. A major band at 40 kD is visible in well 7 of figure 3-14, which is thought to be the expressed recombinant Der p 1. However, on subsequent days of expression, a 25kDa protein band appeared in the culture and the other bands disappeared. This may suggest that the expressed protein is affected by the culture proteases and thus should be harvested at an early stage of expression. The issue of protease degradation in yeast expression systems have been reported in literature; they are thought to be a result of methanol induction and high cell density which triggers excess protease production and cell lysis (Zhang et al., 2007, Sinha et al., 2005). Other optimisation measures in culture conditions are needed if we were to have a consistent hyperglycosylated form. It was reported that the glycosylation of expressed protein in *Pichia* could be controlled via the pH of the medium in addition to controlling the cell density and methanol concentration (Liu H 2005).

In parallel to the expression of the wild type, different mutations to Der p 1 cDNA were done for the glycosylation sites to try and produce different glycoforms. The newly produced glycoforms were assessed by western blot and they all had a 25 kD molecular weight indicating that there was no variation in the

glycosylation pattern and that the factors controlling the production of different glycoform of Der p 1 in *Pichia* are variable and not dependent only on mutating the glycosylation sites alone. The hyperglycosylated form itself was very difficult to be expressed again thus it could not be purified.

As the main aim of expressing Der p 1 was not only to produce different glycoforms but also to have enough quantity of each to get some readouts regarding immune recognition it was a necessity that different methods with more consistent results be introduced to produce the glycoforms from allergens, which chapter four focused on.

CHAPTER FOUR: The role of Der p 1 glycosylation in its uptake by DCs.

4.1 Introduction:

Allergens from the house dust mite *Dermatophagoides pteronyssinus* were first found to be associated with hypersensitivity reactions in 1967 (Spieksma FT, 1967). In 1969, Voorhorst identified the most significant house dust mite allergen, Der p 1 (Voorhorst R, 1969), which later was found to be a major cysteine protease allergen with a proteolytic activity that digests critical cell surface molecules on dendritic cells, T cells and B cells, thus provoking IgE production and allergy (Shakib et al., 2008, Shakib et al., 1998, Voorhorst R, 1969).

It has been shown that up to 90% of individuals who are sensitized to house dust mite allergens are actually responsive to Der p 1 (Sharma et al., 2003, Richardson et al., 2005, Thomas and Smith, 1998). Wan *et al.* found that Der p 1 was able to break the tight junctions of the epithelial cells, suggesting a role for Der p 1 in lung epithelium permeability which contributes to sensitization and allergic responses (Wan et al., 2000). Also, it has been clearly shown that Der p 1 affects both innate and adaptive immune responses. For instance, it induces mast cell and basophil degranulation, which via their

cytokines (e.g. IL-4) influence IgE antibody production. There has been a number of reports showing that Der p 1 can cleave cell surface molecules that control IgE synthesis, like CD25 on T cells, CD23 on B cells and CD40 on DCs (Ghaemmaghami et al., 2002, Shakib et al., 1998). Der p 1 was also shown to cleave DC-SIGN on DCs and DC-SIGNR expressed by endothelial cells (Furmonaviciene *et al.*, 2007). Researchers have shown that proteolytically active Der p 1 suppresses IFN-γ and enhances IL-4 production by both CD4 and CD8 T cells, thus enhancing IgE production by B cells and supporting an overall type 2 cytokine response (Ghaemmaghami *et al.*, 2001). It has also been proposed that cleavage of CD40 on DCs by Der p 1 leads to the production of less IL-12 by DCs. Furthermore, T cells co-cultured with Der p 1 treated DCs produce more IL-4 and less IFN-γ which again directs the immune system towards a Th2 immune response.

Atopic patients mount an exaggerated IgE immune response and tend to polarise a Th2 immune response by producing IL-4, IL-5 and IL-13 (Commins and Platts-Mills, 2010, Kay, 2000). A connection between high levels of thymic stromal lymphopoietin (TSLP) secretion and induction of Th2 immune responses has been established. TSLP is an IL-7-like cytokine expressed by epithelial cells of mouse and human skin, gut, lungs and

thymus. (He and Geha, 2010, Ziegler and Artis, 2010, Wang and Liu, 2009, Soumelis et al., 2002). This cytokine was originally identified in 1994 as a growth factor in culture supernatants of a thymic stromal cell line to support the development of murine immature B cells. Since then, TSLP has been shown to promote B cells, T cells and Th 17 cell homeostasis; it also induces IL-4, 5 and 13 driven allergic inflammation and upregulates MHC I and II co-stimulatory molecules CD40, CD80, CD83 and CD86 (Wang and Xing, 2008, Wang and Liu, 2009, Friend SL, 1994, Ray et al., 1996, Tanaka et al., 2009, Miyata et al., 2009, Soumelis et al., 2002). TSLP can activate DCs and increases their expression of the ligand for T cell costimulatory molecule OX40, thus enhancing Th2 immune responses (Wang and Liu, 2009, Ito et al., 2005, Rochman and Leonard, 2008).

There is a question as to why certain proteins can act as allergens i.e. induce IgE production. The property in proteins that may lead to allergenicity could be enzymatic activity, though not all allergens are enzymatically active. Therefore there could be other common features shared between allergens that contribute to their allergenicity. One feature could be the glycosylation pattern as research on the glycosylation features of allergens have suggested that carbohydrates displayed by allergens could be unique in structure or orientation and thus be recognised by C-type lectin receptors (i.e. the mannose

receptor (MR) on DCs) and this could favour Th2 polarization and allergy (Shakib et al., 1998, Shreffler et al., 2006, Altmann, 2007, Taylor et al., 2005).

The mannose receptor is responsible for the recognition of exposed mannose residues in pathogens and the internalization of mannosylated antigens, thus enhancing their presentation to lymphocytes. MR is composed of 3 extracellular regions: the cysteine rich domain (CR domain) which recognises sulphated sugars, a domain containing fibronectin type two repeats (FNII) and the multiple C-type lectin-like carbohydrate recognition domains (CTLDs) which recognize mannose, fucose and N-acetylglucosamine (Taylor *et al.*, 2005). There is an abundant expression of MR on monocyte derived DCs where MR has been shown to act primarily as an endocytic receptor. Work in our group and that of others suggest that MR on DCs could play a role in the recognition and uptake of Der p 1, and this has been investigated in this chapter through labeling Der p 1 and observing its uptake by DCs. The uptake of Der p 1 can be inhibited by mannan, the natural ligand for MR, and after downregulating the MR expression via gene silencing thus demonstrating the importance of MR in the recognition of Der p 1 (Deslee et al., 2002, Royer et al., 2010).

In recent years a number of different recombinant allergens have been produced which have functional and immunological similarities with their native counterparts. Recombinant allergens were shown to be useful tools in studying allergen specific B and T cell immune responses. It has become increasingly popular to use yeast as a cellular host for the expression of recombinant proteins. *Pichia* is easy to manipulate, faster and less expensive to use than other eukaryotic expression systems, with the advantage of having higher expression levels and the ability to perform post-translational modification like glycosylation (Cereghino and Cregg, 2000, Hamilton and Gerngross, 2007, Jacquet et al., 2002, Yokoyama, 2003). In this chapter, the recombinant preparation of Der p1 that was expressed in *Pichia pastoris* was used as a hyper-glycosylated form of Der p 1 allergen to assess the extent to which glycosylation impacts on Der p 1 uptake and recognition by the immune system.

In an attempt to investigate the role of glycosylation in allergen recognition by the immune system, different glycoforms of Der p 1 were used to detect differences in uptake by DCs. A recombinant preparation of Der p 1 produced in *Pichia was* used as a hypermannosylated form of the allergen. The different Der p 1 glycoforms were prepared using enzymatic and chemical

deglycosylation of the allergen and an assessment of their uptake by MR and DC-SIGN in immature DCs was carried out.

In this chapter, we also used the different glycoforms of Der p 1 produced, to assess any differences binding to murine MR cloned sub-fragment (CTLD4-7-Fc), which is known to bind mannosylated antigens and is shown to have the same specificity as the natural MR sub-fragment (Linehan et al., 2001, Martinez-Pomares et al., 2006).

We also measured changes in secretion of TSLP cytokine by human epithelial cells when exposing them to different glycoforms of Der p 1.

4.2 Materials and methods:

4.2.1 Generation of dendritic cells:

Dendritic cells were generated from peripheral blood-monocytes. Briefly, Heparinized blood from house dust mite-sensitized donors was obtained after prior consent and ethical committee approval. The atopic status of donors was determined by skin prick test (>3mm). Mononuclear cells (PBMC) were separated by standard density gradient centrifugation on Histopaque (HISTOPAQUE-1077, Sigma, Irvine, UK). PBMCs were collected and washed. Purified PBMCs were incubated with mouse anti-human CD14 monoclonal antibody conjugated to magnetic beads (Miltenyi Biotec, Surrey, UK) at 4°C for 20 minutes. Cells

were then washed and applied onto a column placed in the magnetic field of a MACS separator (Miltenyi Biotec, Surrey, UK). Negative cells were eliminated, and the column was removed from the separator and eluted. CD14+ cells (monocytes) were collected and washed with RPMI 1640 (Sigma, Irvine, UK) and their purity was checked. Then they were cultured (1×10^6 cells per well) in 48-well flat-bottomed culture plates (Costar, High Wycombe, UK) in RPMI-1640 medium supplemented with L-glutamine, antibiotics (Sigma, Irvine, UK) and 10% fetal calf serum (FCS, Harlan Sera-Lab, Loughborough, UK) containing 50 ng/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) and 250 U/ml of IL-4 [DC-medium] (R&D Systems, Oxford, UK) at 37°C in 5% CO_2 for 6 days.

On day 3 of culture, fresh DC-medium was added to the cell cultures. On day 6, immature dendritic cells were harvested and used in all the assays described.

4.2.2 Phenotype analysis:

Cells were washed with PBA (PBS, 1% v/v BSA and 0.1% w/v sodium azide) and stained with the appropriate antibodies for 15 min, at 4 ºC in the dark. Cells were then washed twice with PBA and fixed in 0.5% v/v formaldehyde in isotonic azide free solution. Flow cytometry was performed using an EPICS Altra Flow Cytometer (Beckman Coulter, Buckinghamshire, UK). At least 5000 events were collected per sample and isotype

matched antibodies were used to determine binding specificity. The data were analysed using WinMDI version 2.8 (Joseph Trotter, Scripps Institute, La Jolla, CA, USA). Dead cells were excluded from analysis according to their forward and side scatter characteristics.

The following monoclonal antibodies were used: anti-CD11c [Phycoerythrin (PE), clone BU15], anti-CD40 [Phycoerythrin (PE), clone MAB89], anti-CD80 [Fluorescein isothiocyanat, clone MAB104], anti-CD83 [Fluorescein isothiocyanate, clone HB15a], anti-CD86 [Phycoerythrin (PE), clone HA5.2B7], and anti-HLA-DR [Phosphatidylcholine 5 (PC5), clone Immu-357] were all purchased from Coulter Immunotech (Luton, UK).

4.2.3 Enzymatic deglycosylation:

To deglycosylate allergens, different enzymes were used. PNGase A (Roche, Welwyn Garden City, U.K.) was used to hydrolyse N-glycan chains from glycopeptides, specifically α1,3-bound core fucose residues. To hydrolyse 5 µg of Der p 1 50 units of PNGase A were used. Briefly, the reaction was diluted in 50 mM sodium acetate buffer with 5 mM $CaCl_2$ supplemented with 100 µg/ml BSA in 50 µl reaction volume at pH 5.5 and incubated for 6 hrs at 37°C. The reaction was stopped by heat inactivating the enzyme at 70°C for 10 mins. Then, 32 units of

α1-2,3 mannosidase, purchased from New England Biolabs (Hitchin, Uk), was added and the reaction was incubated for another 18 hrs at 37°C, then heat inactivated at 70°C. The other enzyme used was PNGase F (New Englands Biolabs, Hitchin, Uk) at 50 Units, the reaction was supplemented with 1% NP-40, incubated for 6 hrs at 37°C and deactivated at 70°C. Commassie staining Western blot analysis was done to confirm deglycosylation.

4.2.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE):

All protein preparations were analysed by electrophoresis using 12% Novex Tris-Glycine precast gels from Invitrogen. Tris Glycine running buffer (TGS) was prepared (30.3g Tris, 144g Glycine and 10g SDS made up to 1 L with water) and protein samples were run in XCell *SureLock*™ Mini-Cell from Invitrogen under reducing conditions for 1hr 30mins at 125V. Samples were diluted with 4X sample buffer (0.25M Tris-HCl pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue and 0.3M DTT) and heated to 95°C for 5 mins prior to loading. Protein concentrations upon loading were up to 2 µg per well; 5µl of a control pre-stained protein ladder (Fermentas) was added to track sample migration in the first lane of the gel.

4.2.5 Western blot analysis:

After gel electrophoresis, nitrocellulose membranes (Amersham, UK) were cut, soaked in Tris Glycine (TG) blotting buffer (25mM Tris pH8.5, 0.2M Glycine and 20% Methanol), gel and membrane were assembled in an Invitrogen XCell II™ Blot Module apparatus and soaked with 60 ml of TG blotting buffer. The gels were then transferred for 1 hr at 30 V. When finished, the membranes were blocked overnight with 5% BSA in TBS (0.05 M Tris-HCl, 0.15 M NaCl at pH 7.5) at 4°C. In the glycan detection process, the DIG Glycan Differentiation Kit from Roche was used. The membranes were washed twice with TBS and once with buffer 1 (TBS; 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ at pH 7.5). The required amount of lectin solution was added (for GNA 10 µl each; for PNA 100 µl) to 10 ml of buffer 1. These lectins that are Digoxigenin (DIG) labelled were incubated separately for 1 hr with the membranes and the membranes were then washed three times with TBS to remove any unbound antibodies. The secondary antibody was prepared (10 µl of anti-DIG conjugated to Alkaline phosphatise (AP) diluted in 10 ml of TBS) and the membranes were incubated with the antibody for one hour (Fig 4-1). The detection solution was prepared by using 10 ml of Buffer 2 (0.1 M Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl at pH 9.5) diluted in 200 µl of NBT/BCIP solution and the membranes were then incubated with the detection solution for 20 mins in the dark (Colorimetric

detection). The reaction was stopped by washing with ultra pure H_2O. Results were scanned using a BioRad GS800 Densitometer. Positive and negative controls were provided with the DIG Glycan detection kit and were used in the western blot experiments.

4.2.6 Periodate deglycosylation:

Der p 1 preparations were treated with sodium metaperiodate (bought from Sigma-Aldrich inc.) at a molar ratio of 5:1 (Rasheedi et al., 2003, Okana et al., 1999a). The reaction mixture was incubated for 30 and 60 mins at room temperature in the dark. The oxidation process was stopped by adding 0.25 ml ethylene glycol per ml of sample. Samples were then dialyzed at room temperature overnight against PBS. The preparations were then labelled with Lightning-Link FITC Antibody Labeling Kit from Novus Biologicals. Briefly, 1µl of LL-Modifier was added for each 10 µl of allergen to be labeled and was then added to Lightning-Link mix. The reaction was incubated in the dark for 3hrs followed by the addition of 1 µl of LL-quencher reagent for every 10 µl of protein used.

4.2.7 Allergen uptake and inhibition assays:

The MD-DCs were washed and resuspended in uptake medium consisting of RPMI (RPMI 1640, Sigma, Irvine, UK), phosphate buffered saline (PBS) (Sigma, Irvine, UK) and 2% FCS (FCS, Harlan Sera-Lab, Loughborough, UK). Natural Der p 1 (nDer p 1)

preparations were labeled with Cy5 (GE heathcare, Bedford, UK) in some experiments and FITC in others (Novus Bio, Cambridge, Uk), while recombinant Der p 1 (rDer p 1) was labeled with FITC. In the uptake assays cells were pre-incubated with different natural and recombinant Der p 1 preparations (0.5 to 20 µg/ml) and their deglycosylated counterparts (1 µg/ml). In the inhibition assays mannan (200 µg/ml), galactose-PAA (200 µg/ml) and rDer p 1 (0.5 to 20 µg/ml) were incubated with the DC preparations for 20 mins at 37°C before the addition of Der p 1 and incubating for another 25 mins at 37°C. Cells were then washed in uptake medium and the pellets were resuspended in PBS containing 2% FCS.

The quantity of uptake of labeled natural and recombinant Der p 1 was then immediately determined with a Beckman–Coulter Altra flow cytometer (Beckman-Coulter, High Wycombe, UK) and expressed as mean fluorescence intensity (MFI). At least 10,000 cells per sample were analyzed.

4.2.8 Confocal imaging:

Day 6 immature DCs were collected and washed with warm RPMI. rDerp1 (Indoor Biotechnology), labelled with FITC was added at 1.0 µg/ml, N52Q rDer p 1 (an aglycosylated mutant kindly provided from Dr T Takai, Allergy Research Center, Juntendo University School of Medicine, Hongo, Tokyo, Japan) labelled with Alexa 647 (Novus Bio, Cambridge, UK) was added

at 1.0 µg/ml and nDer p 1 labelled with Cy5 (GE heathcare, Bedford, UK) was added also at 1.0 µg/ml to cells. The cells were then incubated at RT for 5, 10 15 and 30 mins, then fixed with 4% formaldehyde and permeabilised with 0.1% triton X. The following antibodies and labelling reagents were used for cell staining, anti-MR (CD206) (PE; Clone 3.29B1.10, Coulter Immunotech) anti-DC-SIGN (CD209) (PE; Clone AZND1, Coulter Immunotech), anti-LAMP-2 PE; Clone GL2A7, Bioquote) ; Fluoro-Trap Fluorescein Labeling Kit [FITC] was used according to manufacturer's protocol (Novusbio, UK). This reaction was incubated for 30 mins at RT, then cells were washed again with PBS and the supernatant was discarded. To label the nucleus, DAPI stain (Thermo scientific) was diluted 1:100 with FBS and 1% BSA and added to all tubes. This reaction was incubated for 5 mins at RT, then cells were washed again with PBS and the supernatant was discarded. Cells were fixed with 0.5% formaldehyde, then the pellet was diluted with 200 µl PBS. Upon imaging, the prepared samples were set up on poly-l-lysine coated slides, covered with cover slips and imaged by LSM 510 meta Confocal Laser Scanning Microscopes (Carl Zeiss int.) at 40X and 60X. Negative controls of cells labelled with the stain only (PE, Cy5, FITC) were used to set up the lasers upon imaging. Co-localization and image analysis were done using the LSM 510 image browser program.

4.2.9 MR binding:

All washes and incubations were carried out in lectin buffer consisting of 10 mM Tris-HCl, pH 7.5, 10mM Ca2+, 0.154M NaCl and 0.05% (w/v) Tween 20. Different Der p 1 glycoforms at concentration of 2µg/ml Der p 1 (Indoor Biotechnology UK), periodate deglycosylated Der p 1 15 mins, periodate deglycosylated Der p 1 30 mins and periodate deglycosylated Der p 1 1 hr, rDer p 1 (Indoor Biotechnology UK), periodate deglycosylated rDer p 1 15 mins, periodate deglycosylated rDer p 1 30 mins, D Der p 1 (an aglycosylated mutant kindly provided from Dr T Takai, Allergy Research Center, Juntendo University School of Medicine, Hongo, Tokyo, Japan), as well as 2 µg/ml of the corresponding carbohydrate ligand [Mannan (Sigma-Aldrich, Irvine, UK) or Galactose (Gal-PAA) (Lectinity, Moscow, Russia)] were used to coat the wells of Maxisorb ELISA plates (Nunc, Roskilde, Denmark) by overnight incubation in PBS at 4°C. The plates were washed three times and the MR subfragment (CTLD4-7-Fc) (kindly provided by Dr Luisa Martinez-Pomares, University of Nottingham, UK) was then added at 2 µg/ml and incubated for 2 hours at room temperature. After three washes, the binding was detected by incubation with anti-human IgG γ-chain-specific alkaline phosphatase conjugate diluted 1:1000 in lectin buffer (Sigma-Aldrich, Irvine, UK). Afterwards, plates were washed three times with lectin buffer and two times with AP

buffer consisting of 100mM Tris-HCl, 100 mM NaCl and 5mM $MgCl_2$ (pH 9.5), and subsequently developed with 100µl/well of (1mg/ml) pNPP (Sigma-Aldrich, Irvine, UK) as a phosphatase chromogenic substrate. Absorbance was measured at 405 nm on a plate reader (Multiskan Ex, Labsystems, Helsinki, Finland). All assays were carried out in triplicate.

4.2.10 Anti-Der p 1 5H8 ELISA:

Different natural unlabelled Der p 1 allergen preparations were used Der p 1 (Indoor Biotechnology UK), periodate deglycosylated Der p 1 15 mins, periodate deglycosylated Der p 1 30 mins and periodate deglycosylated Der p 1 1 hr, rDer p 1 (Indoor Biotechnology UK), periodate deglycosylated rDer p 1 15 mins, periodate deglycosylated rDer p 1 30 mins, D Der p 1 (an aglycosylated mutant kindly provided from Dr T Takai, Allergy Research Center, Juntendo University School of Medicine, Hongo, Tokyo, Japan) at concentration of 2µg/ml, to coat the wells of Maxisorb ELISA plates (Nunc, Roskilde, Denmark) by overnight incubation in PBS at 4°C. The plates were washed three times with TBS tween then 300µl of blocking buffer (TBS, 1% BSA) was added to each well and left for 1 hour. Plates were washed and 5H8 anti-Der p 1 biotinylated antibody (Indoors Biotechnology) was added and incubated at 2µg/ml for 2 hours at room temperature. After three washes, the binding was detected by incubation with Extra Avidin alkaline phosphatase

conjugate diluted 1:1000 in TBS buffer (Sigma-Aldrich, Irvine, UK). Afterwards, plates were washed two times with TBS tween buffer and subsequently developed with 100µl/well of (1mg/ml) pNPP (Sigma-Aldrich, Irvine, UK). Absorbance was measured at 405 nm on a plate reader (Multiskan Ex, Labsystems, Helsinki, Finland). All assays were carried out in triplicate.

4.2.11 Cell culture:

Epithelial cell lines representing bronchial human lung (BEAS-2B) were kindly provided by Professor Ian Hall (University of Nottingham, UK). They were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen), along with 10% low endotoxin foetal bovine serum (Autogen Bioclear, UK), 2mM L-Glutamine (Sigma) and 1% Penicillin/Streptomycin (Sigma). Cells were cultured until 80% confluence, after which spent media was aspirated and flasks were washed with 1x PBS. The cells were then trypsinised using 0.25% trypsin-EDTA (Sigma) and incubated for 5 minutes at 37°C, 5%CO_2. According to the number of cells to be re-passaged, cells were spun down for 5 minutes at 300 RPM. The resulting cell pellets were then re-suspended thoroughly in the appropriate media and transferred to T75 flasks (Corning) for culturing. The number of cells used for each condition was 1 x 10^6 cells/ml. A cell count was performed using a haemocytometer after staining with 0.1% Trypan blue solution (Sigma) to exclude dead cells.

4.2.12 Co-culturing BEAS-2B epithelial cells with different Der p 1 glycoform preparations:

BEAS-2B cells were added to 24-well plates (Corning), together with either 1µg/ml of Der p 1 (Indoors Biotechnology), periodate deglycosylated Der p 1 (1µg/ml), rDer p 1 (Indoors Biotechnology) (1µg/ml) or periodate deglycosylated rDer p 1 (1µg/ml). Then LPS (sigma) was added to all conditions at 50ng/ml. For some experiments human normal lung bronchial fibroblasts provided by City Hospital (Nottingham, UK) were co-cultured with epithelial cells for 48 hrs until confluent. Plates were then incubated at 37°C, 5% CO_2 for 24 hours. At the end of incubation, supernatants were carefully collected from wells and transferred to sterile 1.5ml eppendorf tubes (Axygen), then frozen at -20ºC.

4.2.13 TSLP ELISA:

Levels of human TSLP (hTSLP) in epithelial cell culture supernatants were measured with a Human TSLP ELISA development kit, which was purchased from Biolegend and can detect human TSLP concentration to a minimum of 4pg/ml. Briefly, 96-well plates (Nunclon) were coated with TSLP capture antibody diluted 1:200 from 200X stock solution and incubated overnight. All incubations were carried out at room temperature, and plates were aspirated and washed 4 times with wash buffer (0.05%Tween-20 in 1xPBS, Sigma) between steps. Following

overnight incubation, 300µl of blocking buffer provided with the kit was added to each well and left for 1 hour. Then, 100µl of samples and standards (prepared by serial dilutions to obtain a range of 7.8 - 500pg/ml) were added to appropriate wells and incubated for 2 hours. Following this, the detection antibody was added and incubated for another 1 hour. The plate was washed 4 times then 100µl of Avidin-HRP conjugate that is provided by the kit was added to each well and left for 30 minutes. Finally, 100µl per well of substrate solution F (Biolegend) was added and incubated in the dark for 15 mins, then the reaction was stopped by adding 100 µl of stop solution to each well. The plate was read by an ELISA plate reader (Labsystems, Multiskan EX) at a wavelength of 450nm.

4.2.14 CD4+ T cell separation:

Using the Pan T cell separation kit (Miltenyi Biotec, Surrey, UK), the negative fraction collected after separating CD14+ cells from PBMC was washed with RPMI 1640 (Sigma, Irvine, UK), then human T cells were isolated by depletion of non-T cells (negative selection). Non-T cells are indirectly labeled with a cocktail of biotin conjugated monoclonal antibodies (CD14, CD16, CD19, CD36, CD56, CD123, and CD235a) as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. The magnetically labeled non-T cells are depleted by retaining them on a MACS® Column in the

magnetic field of a MACS Separator, while the unlabeled T cells pass through the column. The purity of the enriched T cell fraction was evaluated by flow cytometry against CD3 FITC antibody. Cells were then washed by MACS buffer, centrifuged and resuspended in 500µl of buffer. Then using the CD4 naive T cell isolation kit (CD45RO microbeads from Miltenyi Biotec, Surrey, UK), human DC4+ T cells were isolated by depletion of non-CD4+ T cells (negative selection). Again, non-CD4+ T cells are indirectly labeled with CD45RO biotin conjugated monoclonal antibody, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. The magnetically labeled non-CD4+ T cells are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled CD4+ T cells pass through the column. The purity of the enriched CD4+ T cell fraction was evaluated by flow cytometry.

4.2.15 DC-CD4+ T cell Co-Culture:

The immature DCs generated were collected at day 6 and activated by the enriched TSLP supernatant produced by culturing BEAS-2B epithelial cells with different Der p 1 glycoform preparations, in addition to recombinant TSLP standard (Biolegend, UK). For each condition, 2 x 10^5 DCs were used and 500µl of TSLP supernatant was added. All the conditions were incubated for 6 hours at 37°C, 5%CO_2, then the

supernatant was removed and the cells were washed thoroughly with warm RPMI 1640 (Sigma, Irvine, UK).

The activated DCs were then co-cultured with naive CD4+ T cells for 7 days at a ratio of 1:1 in a round bottomed 96-well culture plates. In some experiments immature DCs were directly stimulated with different glycoforms of Der p 1 and recombinant Der p 1 for 24 hrs then co-cultured with CD4+ naive T cells for 10 days. The cytokine secretion for IL-4 and IFN-γ was then measured. Medium used for the co-culture was RPMI 1640 supplemented with penicillin/streptomycin and 5% human AB serum (Sigma-Aldrich). On day 3, the culture was fed with IL-2 (20 IU/ml; R&D Systems). At day 7, T cells were re-stimulated for 8 h with PMA (15 ng/ml) and ionomycin (1mg/ml) (both from Sigma-Aldrich). The supernatants were collected and stored at -20°C.

4.2.16 Cytokine measurement:

Cytokine secretion was analysed with the FlowCytomix kit (Bender MedSystems Vienna, Austria) according to manufacturer's instructions.

4.2.17 Statistical analysis

Statistical analysis of the data was carried out using Student's t-test because of the small number of replicates being used, *P*-

values < 0.05 were considered significant. Data were expressed as the MFI ± SDM, number of independent experiments ≥3.

4.3 Results:

4.3.1 Comparative analysis of natural and recombinant Der p 1 uptake by immature DCs:

Both natural and recombinant Der p 1 preparations are glycosylated (see chapter 2). In this project we proved that the recombinant preparation is hyperglycosylated and has much more mannan than the natural Der p 1. To investigate the effect of hyperglycosylation on the uptake of Der p 1 allergen by DCs we incubated both preparations under the same conditions *in vitro* with immature DCs at 37°C. Both preparations were labelled by FITC and thus the uptake could be measured comparatively by flow cytometry as MFI readings. The control conditions for these experiments were DCs incubated with allergens at 4°C and DCs only. Levels of allergen uptake for each condition is presented as MFI (Fig 4-1) and is also visualised using confocal imaging (Fig 4-2). The results suggest that the average mean of uptake for the recombinant Der p 1 (hyperglycosylated allergen) is significantly higher than that of the natural one. To investigate the effect of deglycosylation of allergens on their uptake N52Q was used, which is a deglycosylated preparation of recombinant Der p 1, it showed a

lower uptake compared to the recombinant hyperglycosylated preparation (Fig 4-3). In other experiments the uptake of natural Der p 1 was blocked by rDer p 1 in a dose dependent manner (Fig 4-4). This inhibition of nDer p 1 uptake reached 62.5% upon adding 20ug/ml rDer p 1 preparation to the DCs. This was also confirmed by confocal imaging (Fig 4-5).

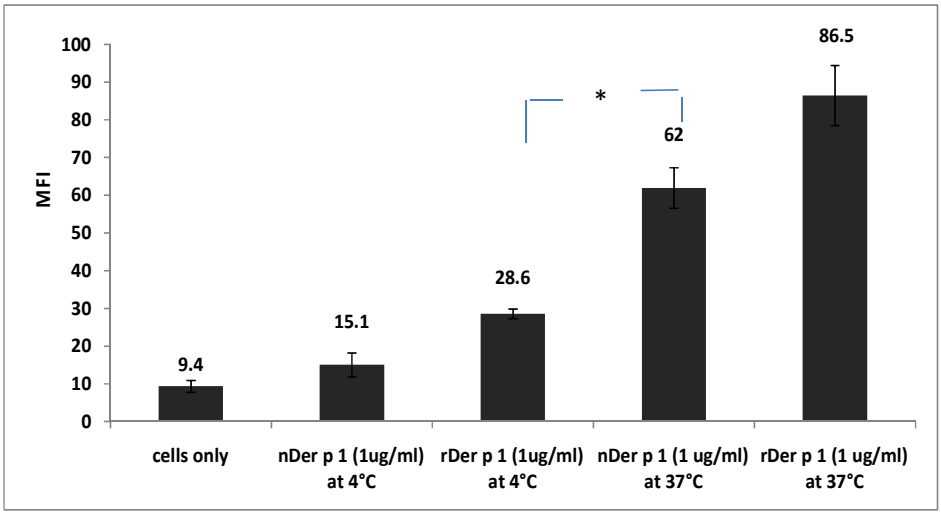


Fig 4-1: MFI ± SDM readings which represent the difference in uptake between natural and recombinant Der p 1 (1µg/ml) by immature DCs. There was a significant difference between native and recombinant uptake at the same concentration. The results suggest that the average mean of uptake for the recombinant preparation is higher than that for the natural one, the results also show that the uptake of Der p 1 by immature DCs at 4°C is lower than the uptake at 37°C for both preparations. Both natural and recombinant Der p 1 were labelled with FITC and

incubated at 4°C and 37°C for 20 mins with DCs . *P *value*<0.05, number of independent experiments=3.

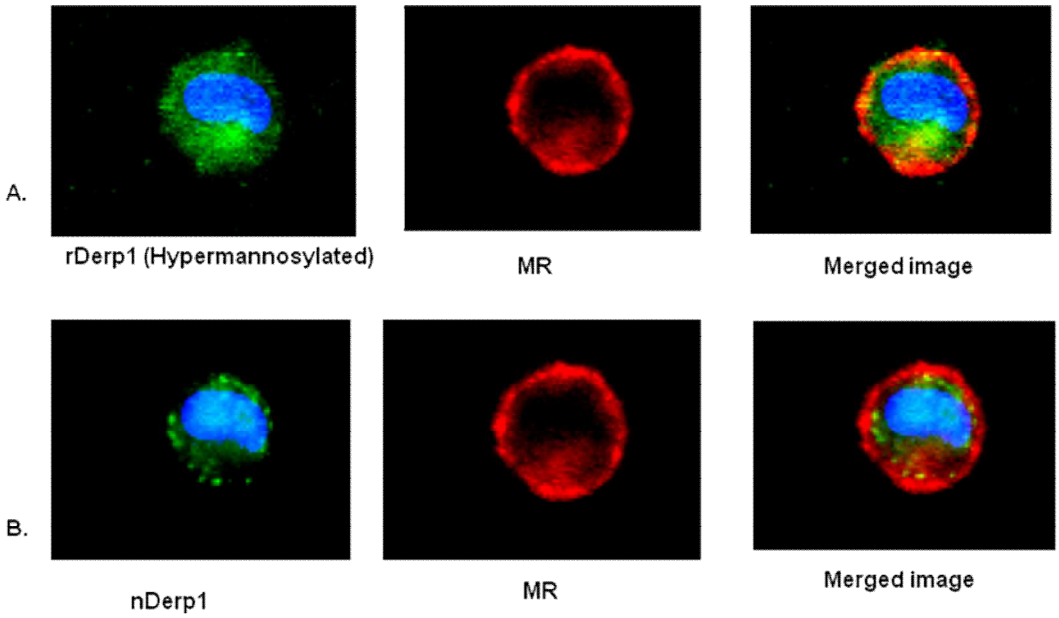


Fig 4-2: Confocal images of the difference between recombinant and natural Der p 1 (0.5 µg/ml) uptake by immature DCs. The results suggest that the uptake of the recombinant preparation (A) is higher than that for the native one (B) in the same DC. A. Green:rDer p 1 labelled with FITC, red:MR labelled with PE, blue:nucleus labelled with DAPI. B. Green:nDer p 1 Labelled with Cy5, red:MR labelled with PE, blue:nucleus labelled with DAPI, number of independent experiments=3.

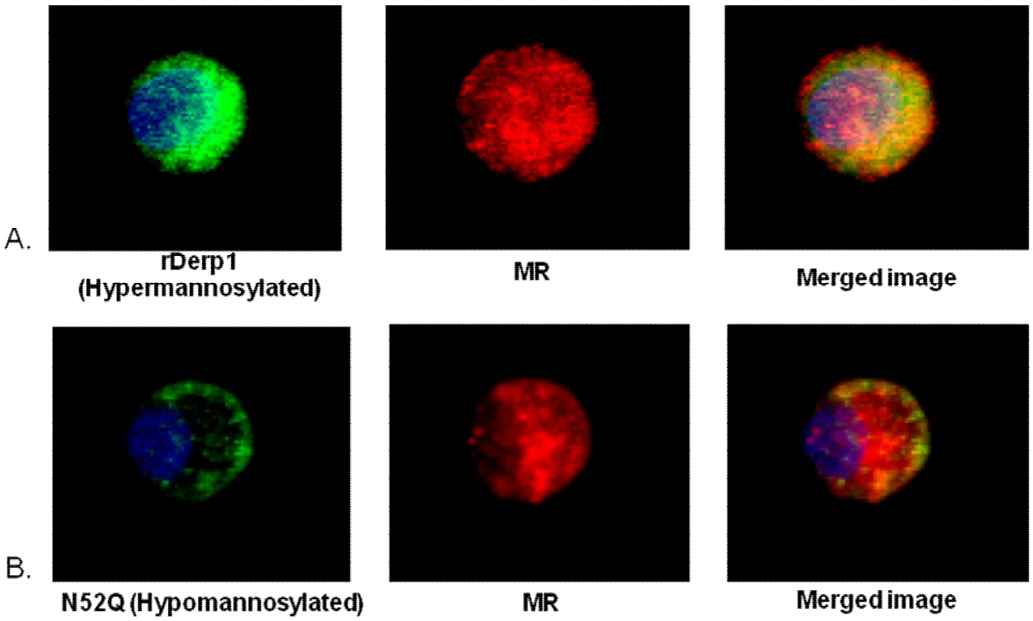


Fig 4-3: Confocal images of the difference between recombinant Der p 1 and recombinant N52Q Der p 1 (0.5 μg/ml) uptake by immature DCs. The results suggest that the uptake of the recombinant hypermannosylated Der p 1 preparation (A) is higher than that for the recombinant hypomannosylated Der p 1 (N52Q) (B) in DCs. A. Green:rDer p 1 labelled with FITC, red:MR labelled with PE, blue:nucleus labelled with DAPI.B. Green:N52Q Der p 1 Labelled with Alexa 647, red:MR labelled with PE, blue:nucleus labelled with DAPI, number of independent experiments=3.

When examining the uptake of recombinant and natural Der p 1 preparations at different timepoints by confocal imaging, there seems to be a different pattern in uptake i.e. rDer p 1 is diffused whereas native Der p 1 is localised on cell memberane (Fig 4-2 & 4-3). The recombinant preparation seems to be internalized

immediatly by DCs after 5 mins incubation while the natural preparation only started to be taken up at 10 mins (Figs 4-6 & 4-7). The uptake of both preparations was studied at 20 and 30 mins (Fig 4-8 & 4-9). These images also show the co-localisation of both preparations by MR. When analysing the co-localization of natural and recombinant preperations with MR by the LSM 510 image browser, the co-localization coeffecient was found to be higher for rDer p 1 (Figs 4-10 & 4-11). The analysis also showed that these preparations co-localise inside the cell suggesting a similar intracellular fate (Fig 4-12). Recombinant and natural Der p 1 also co-localised with DC-SIGN (Fig 4-13) and with the Lysosomal-associated membrane protein 2 (LAMP-2), which shuttles between lysosomes, endosomes and the plasma membrane (Fig 4-14).

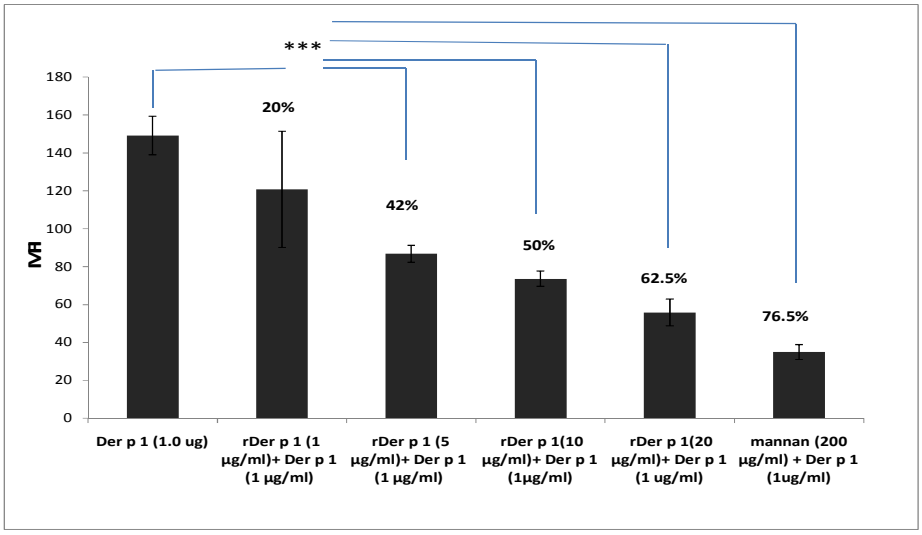
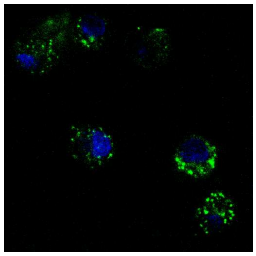
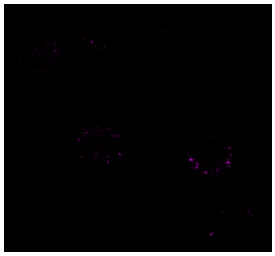
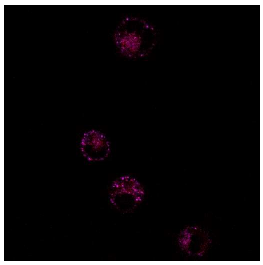


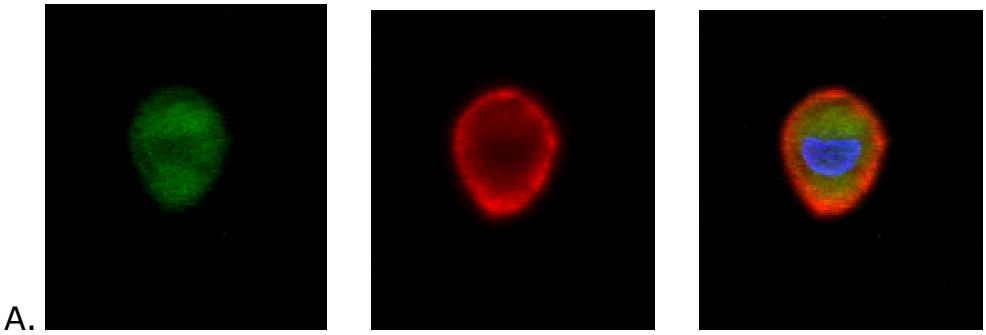
Fig 4-4: natural Der p 1 uptake by immature DCs represented as MFI ± SDM readings when adding different concentrations of rDer p 1 to the DCs (up to 20µg/ml) and mannan (200µg/ml). There was a significant decrease in uptake of natural Der p 1 when comparing the preparations before and after adding rDer p 1; after adding 5 µg/ml of rDer p 1 to the DCs then adding 1µg/ml concentration of natural Der p 1, the uptake of natural Der p 1 was decreased by 42% on average compared to the natural uptake alone at 1µg/ml, this inhibition of uptake is dependent on rDer p 1 addition concentration as the higher the concentration of rDer p 1 the more the Der p 1 uptake is inhibited. The inhibition of natural Der p 1 uptake reached 62.5% when adding 20µg/ml of rDer p 1 .Natural Der p 1 was

labelled with Cy5, rDer p 1 was not labelled.*** *P value*<0.001. number of independent experiments=3

A.  B.  C. 

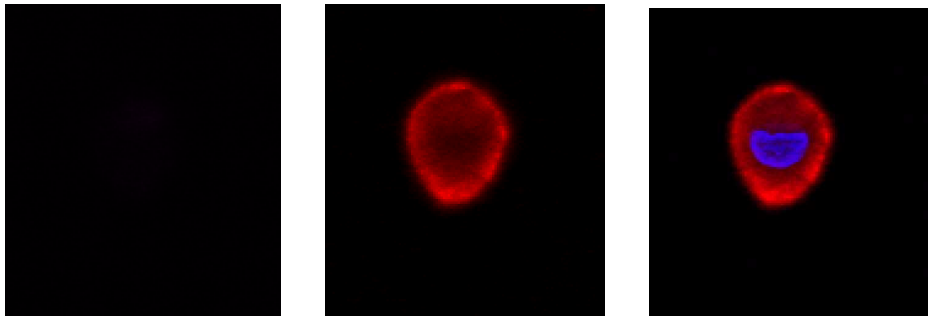
rDer p 1 nDer p 1 Control of uptake of nDer p 1

Fig 4-5: In A & B the uptake of nDer p 1 at 1µg/ml by the same immature DCs when adding 10 µg/ml of rDer p 1. C. Is the control of uptake of n Der p 1 when no rDer p 1 is added. The images suggest that the uptake of nDer p 1 (1µg/ml) (B) is inhibited after adding (10µg/ml) of rDer p 1 (A) in the same DCs. A. Green: rDer p 1 stained with FITC, B. purple: nDer p 1 stained with Cy5, C. Purple: Control slide of nDer p 1 stained with Cy5 (1µg/ml) uptake when no rDer p1 added, number of independent experiments=3.



A.

rDer p1 MR Merged image



B.

nDer p 1 MR Merged image

Fig 4-6: The uptake of recombinant and natural preparations of Der p 1 (1 μg/ml) by immature DCs at 5 mins. The image shows that at 5 mins the recombinant preparation is inside the DCs while the natural is not. A. Green: rDer p1 stained with FITC, red:MR stained with PE, blue: nucleus stained with DAPI, B. Green: nDer p 1 stained with Cy5, red: MR stained with PE, blue: nucleus stained with DAPI, number of independent experiments=3.

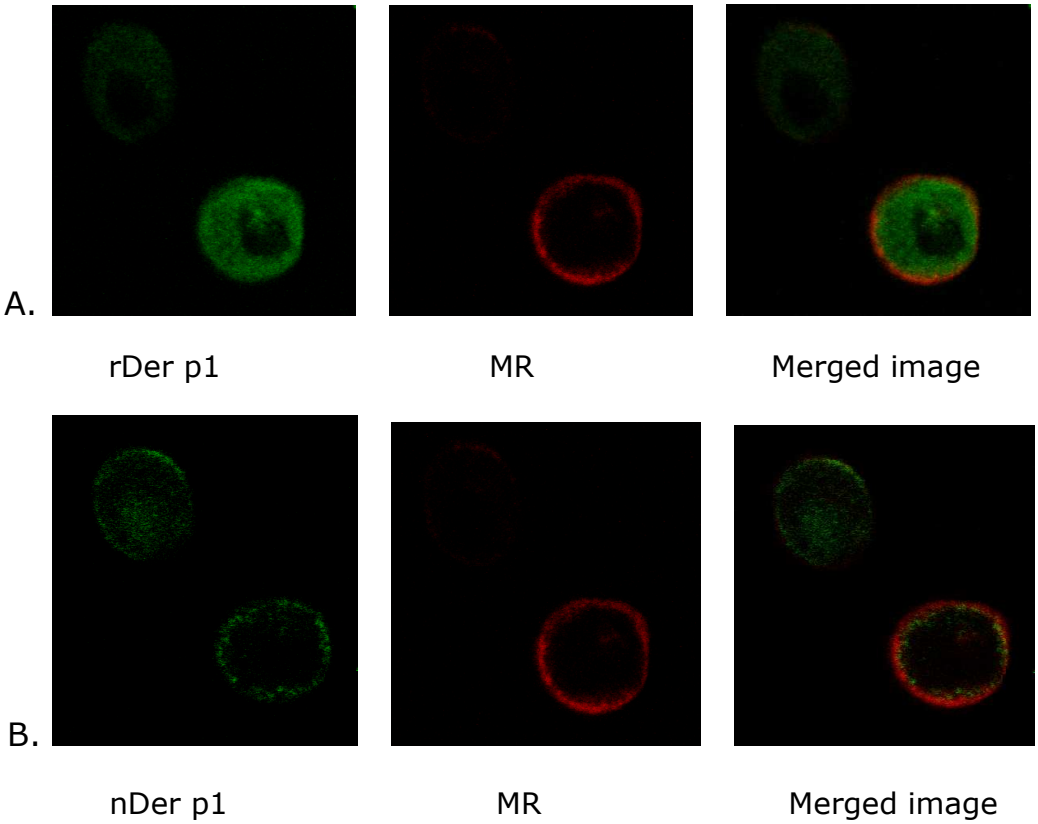
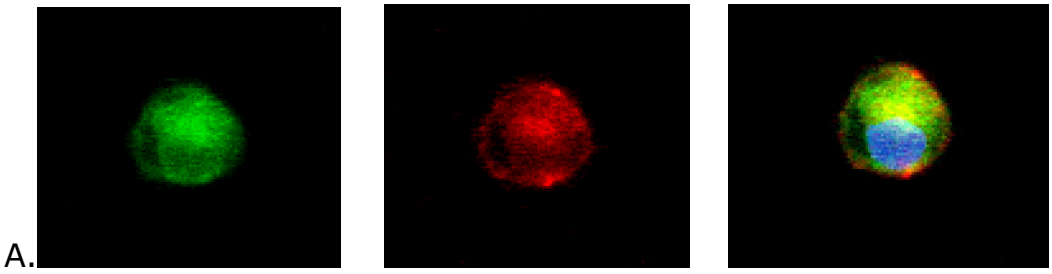
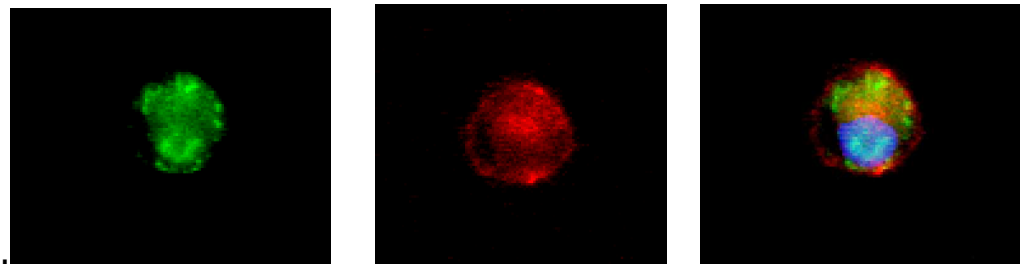


Fig 4-7: The uptake of recombinant and natural preparations of Der p 1 (1 μg/ml) by immature DCs at 10 mins. The image shows that at 10 mins both preparations are inside the DCs A. Green: rDer p1 stained with FITC, red: MR stained with PE, B. Green: nDer p 1 stained with Cy5, red: MR stained with PE, number of independent experiments=3.



A.

rDer p1 MR Merged image



B.

nDer p1 MR Merged image

Fig 4-8: The uptake of recombinant and natural preparations of Der p 1 (1 μg/ml) by immature DC at 20 mins. A. Green: rDer p1 stained with FITC, red: MR stained with PE, blue: nucleus stained with DAPI. B. Green: nDer p 1 stained with Cy5, red: MR stained with PE, blue: nucleus stained with DAPI, number of independent experiments=3.

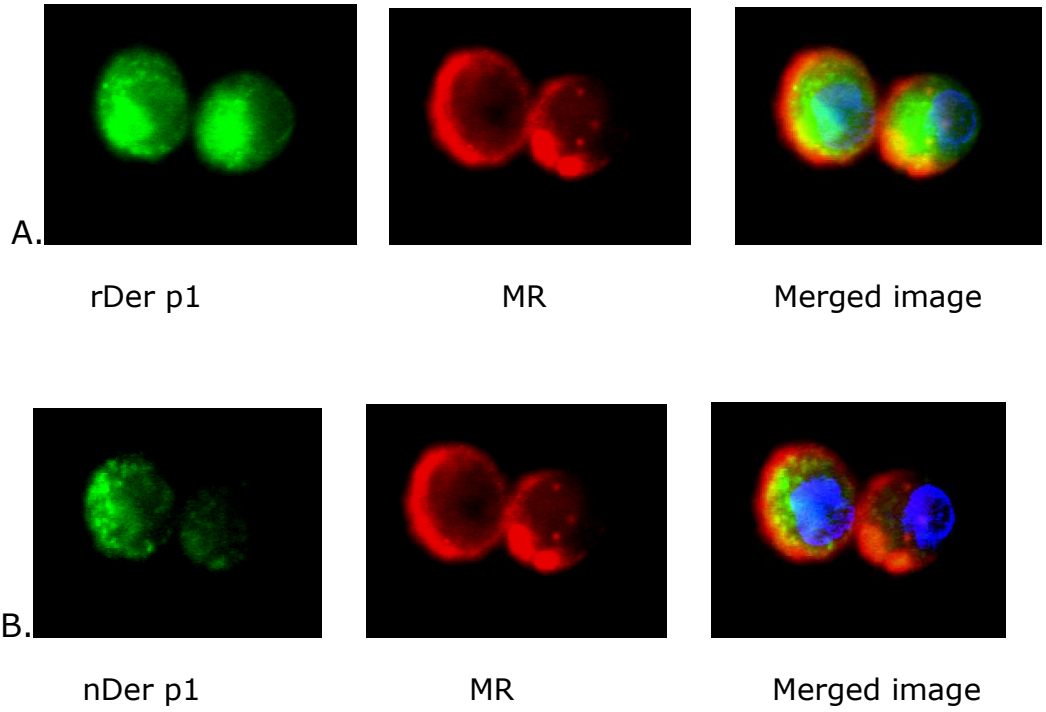


Fig 4-9: The uptake of recombinant and natural preparations of Der p 1 (1 μg/ml) by immature DCs at 30 mins. A. Green: rDer p1 stained with FITC, red: MR stained with PE, blue: nucleus stained with DAPI. B. Green: nDer p 1 stained with Cy5, red: MR stained with PE, blue: nucleus stained with DAPI, number of independent experiments=3.

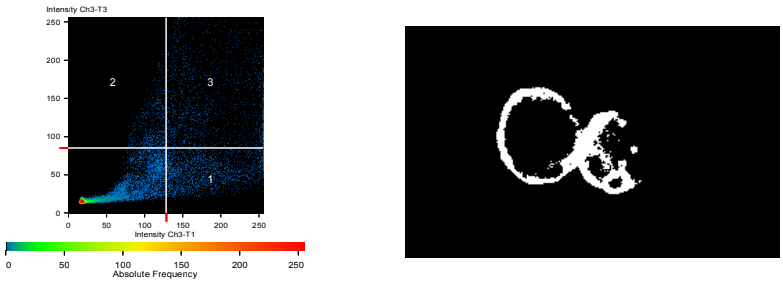


Fig 4-10: rDer p 1 (FITC labelled) co-localization analysis with MR (PE labelled) using the LSM image browser 510, Co-localization coeffcient: 0.911, Overlap coefficient:1.0. The results indicate that there is co-localisation between the MR and rDer p 1 inside the DC.

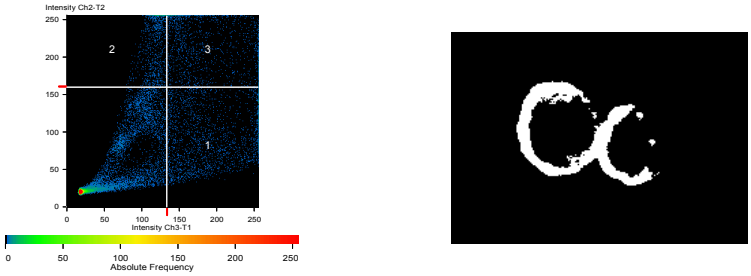


Fig 4-11: nDer p 1 (Cy 5 labelled) co-localisation anlysis with MR (PE labelled) using the LSM image browser 510. Co-localization coeffcient: 0.84, Overlap coefficient:0.9. The results indicate that there is co-localisation between the MR and nDer p 1 inside the DC.

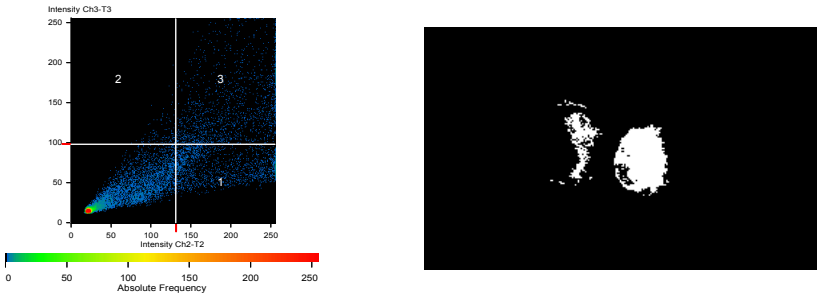
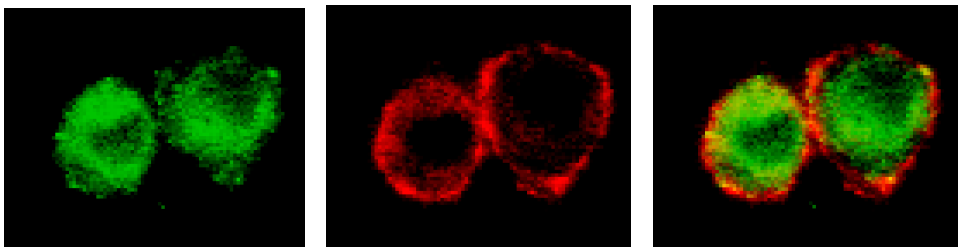
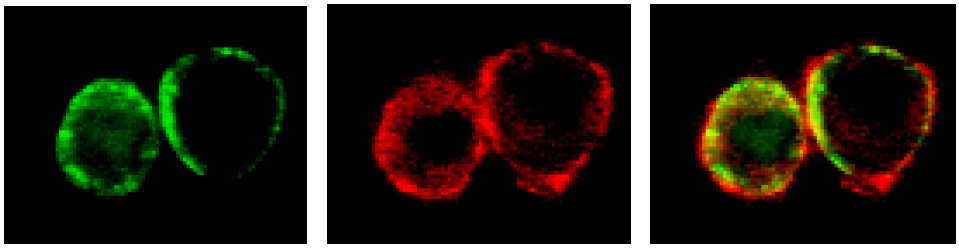


Fig 4-12: nDer p 1 (Cy 5 labelled) co-localisation anlaysis with rDer p 1 (FITC labelled) using the LSM image browser 510. Co-localization coefficient:0.73, Overlap coefficient:1.0. The results indicate that there is co-localisation between the nDer p 1 and rDer p 1inside the DC.



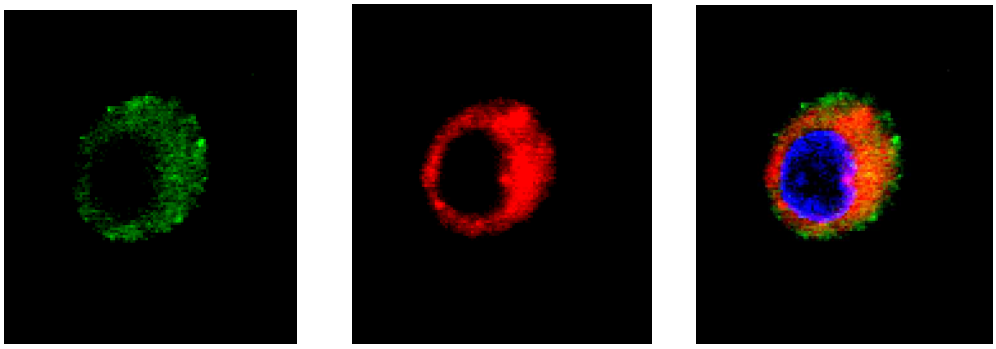
rDerp1 DC-SIGN Merged image



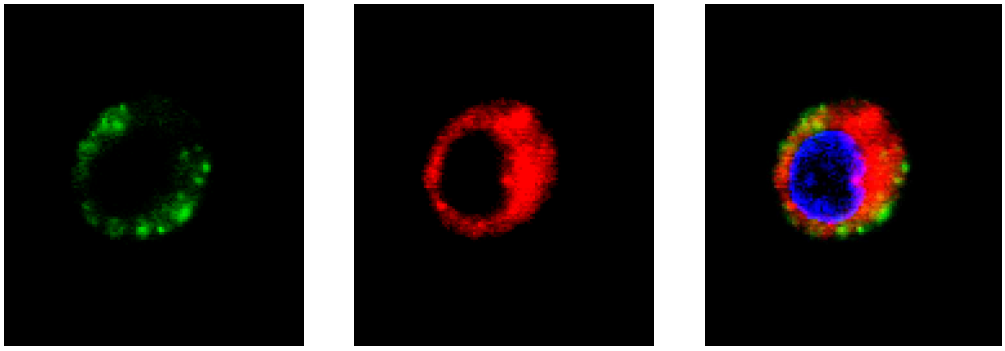
nDerp1 DC-SIGN Merged image

Fig 4-13: The co-localisation of native and recombinant Der p 1 (0.5 µg/ml) in immature DCs with DC-SIGN detected at 10 mins. rDer p 1 co-localization coeffcient with DC-SIGN:0.83, nDer p 1

Co-localization coeffcient with DC-SIGN:0.75. A. Green: rDer p1 stained with FITC, red: DC-SIGN stained with PE, B. Green: nDer p 1 stained with Cy5, red: DC-SIGN stained with PE, number of independent experiments=3.



rDerp1 LAMP-2 Merged image



nDerp1 LAMP-2 Merged image

Fig 4-14: The co-localisation of native and recombinant Derp1 (0.5 μg/ml) with LAMP-2 detected at 10 mins. rDer p 1 Co-localization coeffcient with LAMP-2:0.88, nDer p 1 Co-localization coeffcient with LAMP-2:0.71. A. Green: rDer p1 stained with FITC, red: MR stained with PE, blue: nucleus stained with DAPI. B. Green: nDer p 1 stained with Cy5, red: MR

stained with PE, blue: nucleus stained with DAPI. number of independent experiments=3.

4.3.2 Enzymatic deglycosylation of natural and recombinant Der p 1:

In order to get different glycoforms of the same Der p 1 preparation and investigate the difference between their recognition by the immune system, enzymatic deglycosylation was used to try to specifically demannosylate the different allergen preparations. Three enzymes were used to achieve minimal glycosylation levels of natural and recombinant Der p 1 preparations. We used PNGase A that digests N-glycan chains, in particular the α1,3-bound core fucose residue. We also used 1,3 and 1,2 mannosidase and PNGase F that cleave between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. Examining allergen preparations after deglycosylation showed that the molecular weight of the recombinant Der p 1 preparation dropped from 37kD to around 25kD and it no longer migrated as a diffuse band (Fig 4-15, well 9). This suggests that a lot of sugars were broken down as they appear as a band at 10kD in well 9. The natural Der p 1 preparation did not show a drop in molecular weight and still migrated as a diffuse band, which suggests that either the demmanosylation process did not work or that there was only a

small amount of mannan present on the protein and most of the glycosylation in Der p 1 is due to other sugars like galactose (see chapter 2).

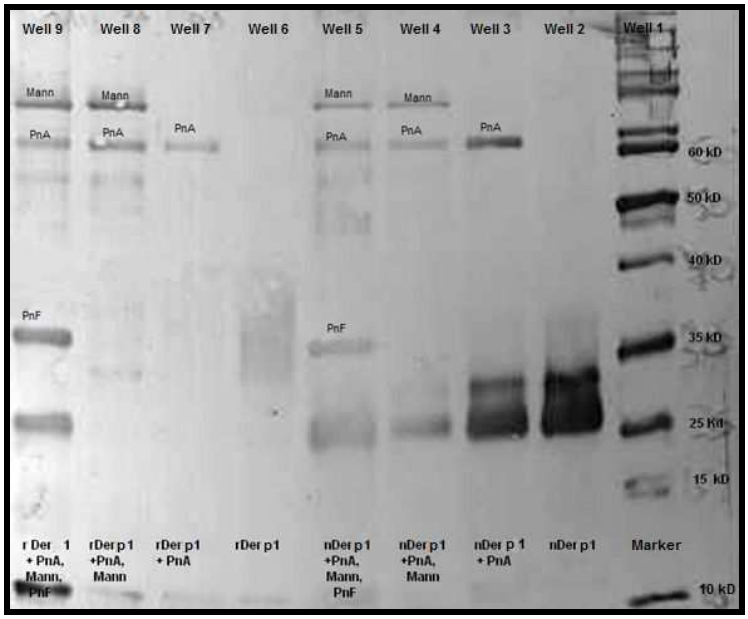


Fig 4-15: Deglycosylation of natural and recombinant Der p 1 with PnGase A (PnA), 1,2 1,3 Mannosidase (Mann) and PNGase F (PnF). Well 1:marker, well 2:nDer p 1, well 3: nDer p 1 digest with PnA, well 4:nDer p 1 digest with PnA and Mann, well 5:nDer p1 digest with PnA, Mann and PnF, well 6: r Der p 1, well 7: rDer p 1 digest with PnA, well 8:rDer p 1 digest with PnA and mannosidase, well 9: rDer p 1 after deglycosylation with the PnA, Mann and PnF enzymes. This is a blot against anti-galactose PNA , number of independent experiments=2.

The demannosylated recombinant Der p 1 preparation was labelled with FITC and its uptake by DCs was compared to the

hypermannosylated allergen using flowcytometry (Figs 4-16 & 4-17). The results indicate a significant decrease in the uptake of demannosylated Der p 1 by immature DCs compared with the untreated allergen. Pre incubation of DCs with mannan (200ug/ml) prior to addition of untreated recombinant Der p 1 preparation resulted in significant inhibition in its uptake by 88%. This result was also confirmed by confocal imaging (Fig 4-18). This experiment confirmed the importance of mannan in the uptake of rDer p 1 allergen.

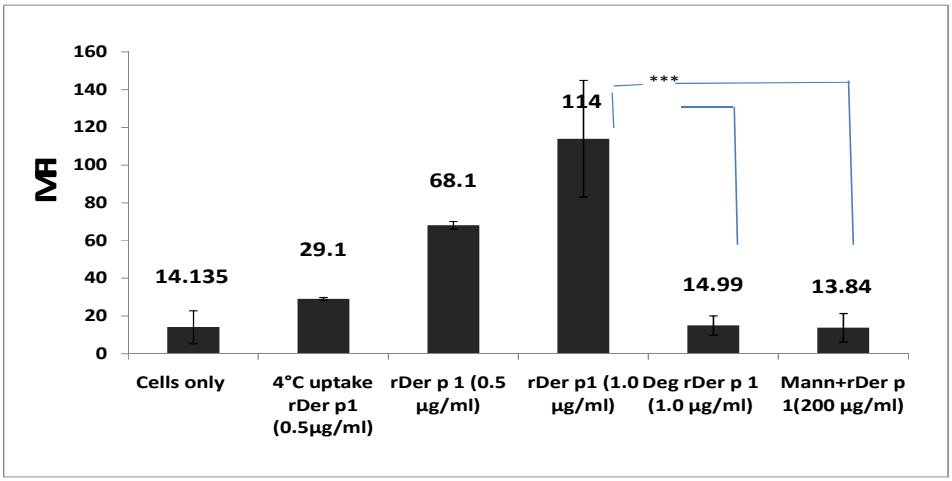


Fig 4-16: The MFI ± SDM readings for the uptake of different concentrations of rDer p 1 by immature DCs compared to enzymatically deglycosylated rDer p 1 using PNGase A, mannosidase and PNGase F, the results indicate a significant decrease in the uptake of demannosylated rDer p 1 by immature DCs compared with the undigested allergen. ***P

value<0.001, rDer p 1 was labelled with FITC. number of independent experiments=3

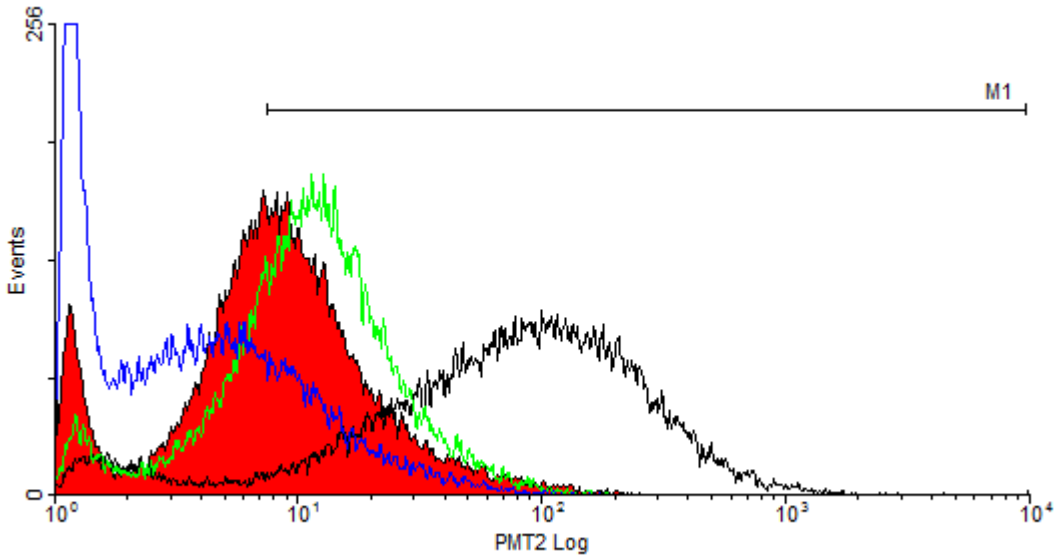
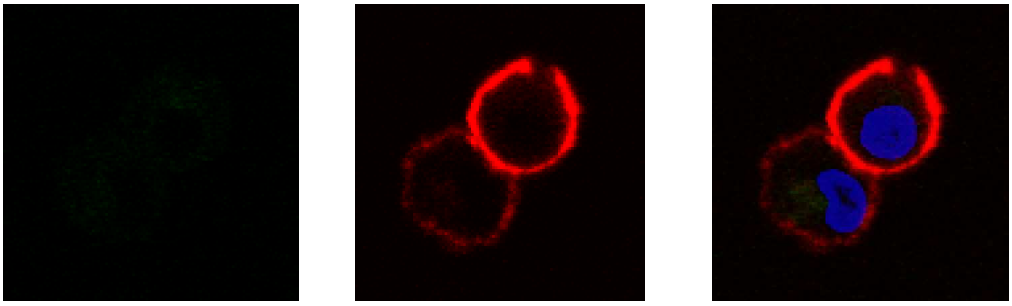
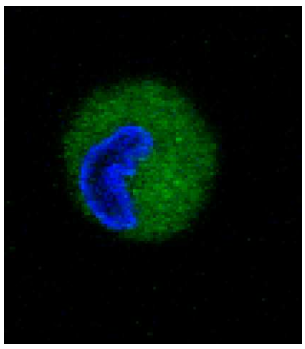


Fig 4-17: DC populations expressed as histogram by the WIN MDI 2.9 program after the uptake of different rDer p1 preparations. Red: cells only, Black: rDer p 1 1ug/ml, green: demannosylated rDer p 1 1µg/ml, blue: mannan 200 µg/ml+rDer p 1 1µg/ml.

A.

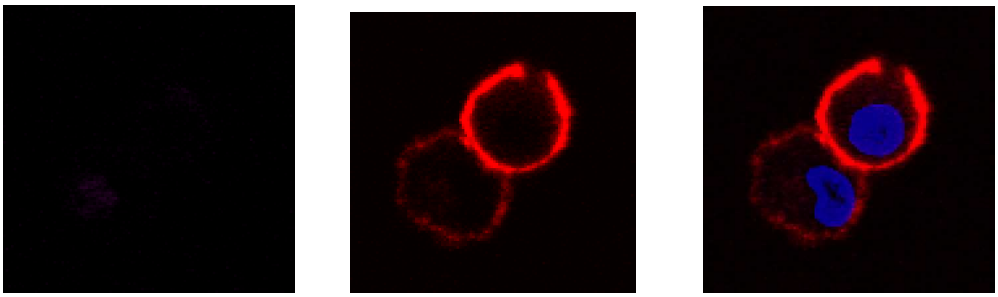


Mannan+ rDer p 1 MR Merged image

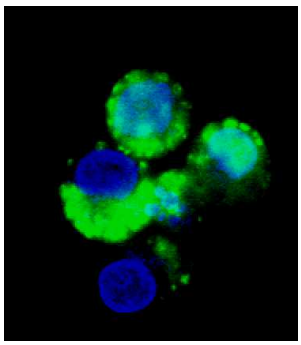


Control of the uptake of rDer p 1 (Green) by DCs without the addition of mannan

B.



Mannan+ nDer p 1 MR Merged image



Control of the uptake of nDer p 1 (Green) by DCs without the addition of mannan

Fig 4-18: Confocal image showing the inhibition of uptake of rDer p 1 and nDer p 1 when adding mannan to the cells at 200

µg/ml. The recombinant and natural Der p 1 concentration used is 1 µg/ml. The results shows that there is minimal uptake for both preparations upon adding mannan to the immature DCs compared to previous results with no mannan addition. A. Green: rDer p1 stained with FITC, red: MR stained with PE, blue: nucleus stained with DAPI. B. Green: nDer p 1 stained with Cy5, red: MR stained with PE, blue: nucleus stained with DAPI, number of independent experiments=3.

4.3.3 Sodium periodate deglycosylation of native and recombinant Der p 1:

Another method was used to try and deglycosylate both native and recombinant Der p 1 preparations by using sodium metaperiodate oxidation. Periodate has been used in the literature to deglycosylate protein preparations (Perlin, 2006, Lipniunas et al., 1992, Philip E. Thorpe, 1988, Okano et al., 1999). Natural and recombinant Der p 1 were incubated with periodate in the dark at room temperature for 30 and 60 mins. A western blot against GNA (anti 1-2,3,6 mannose was performed on the samples before and after periodate treatment (Fig 4-19) to confirm that demannosylation had worked.

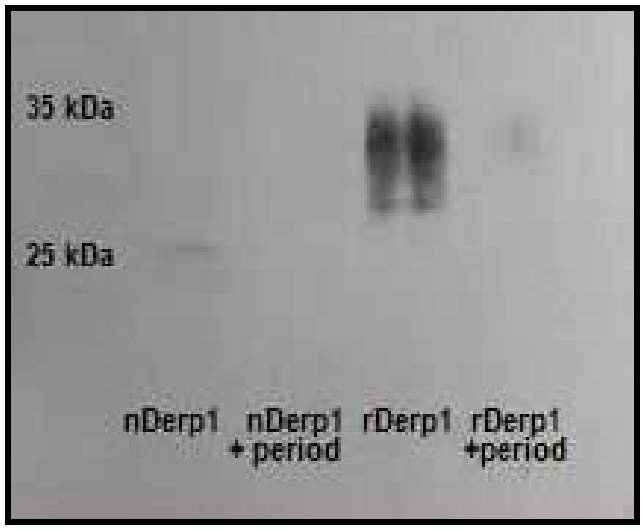


Fig 4-19: Western blot against GNA (anti-mannose) of natural and recombinant Der p 1 before and after periodate treatment for 60 mins. The blot shows minimal reaction with GNA for both preparations after periodate treatment, indicating that periodate removed most of mannan. The concentration of the protein loaded in each well was 2.0 µg/ml, number of independent experiments=2.

Preparations were then labelled with FITC and the uptake by DCs was measured against untreated preparations (Figs 4-20 & 4-21).

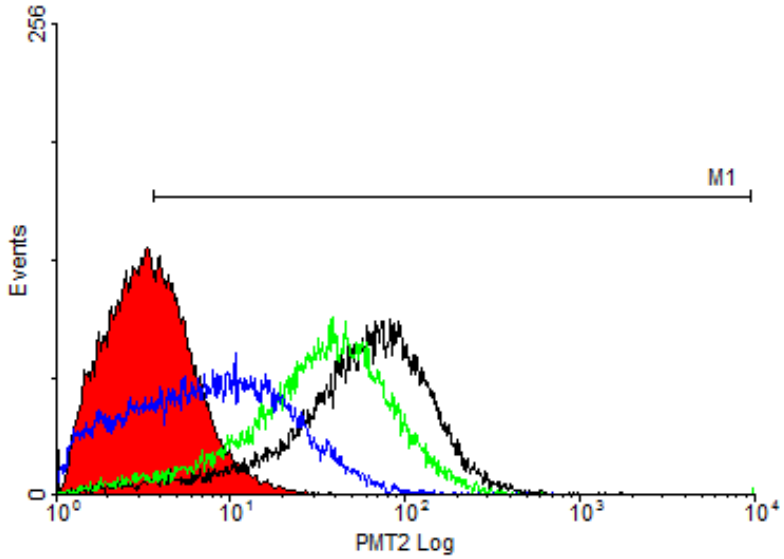


Fig 4-20: Uptake of different Der p 1 preparations by immature DCs. Red:cells only, Black: 0.5 µg nDer p 1 , green :30 mins periodate of 0.5 µg nDer p 1, blue: 1 hr treatment of nDer p 1 (1 µg/ml).

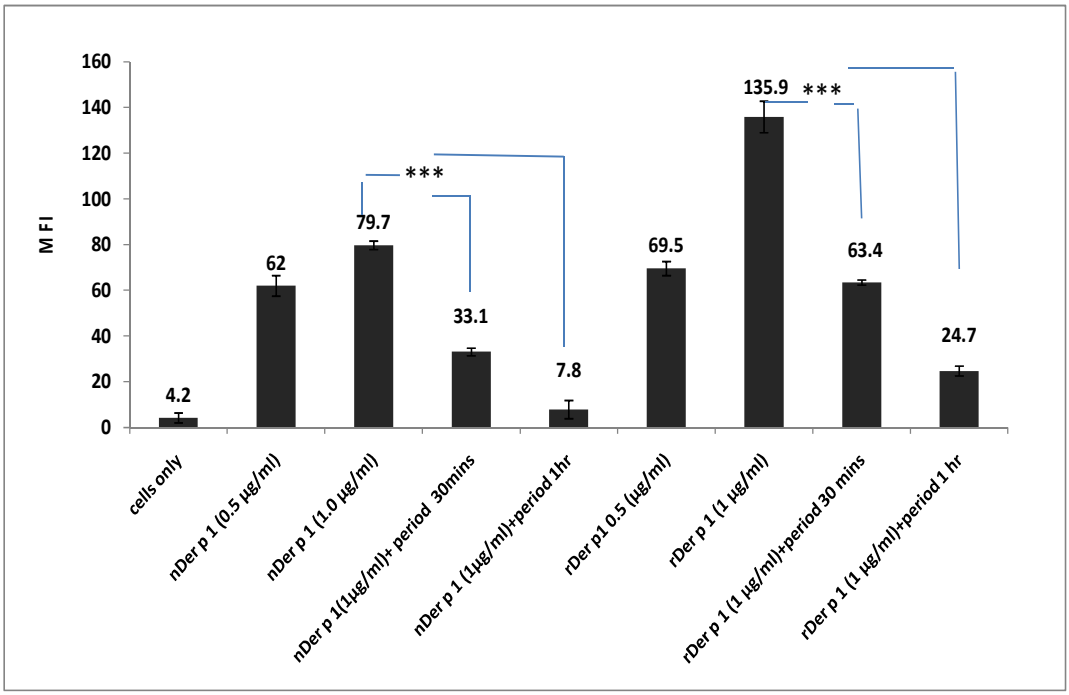
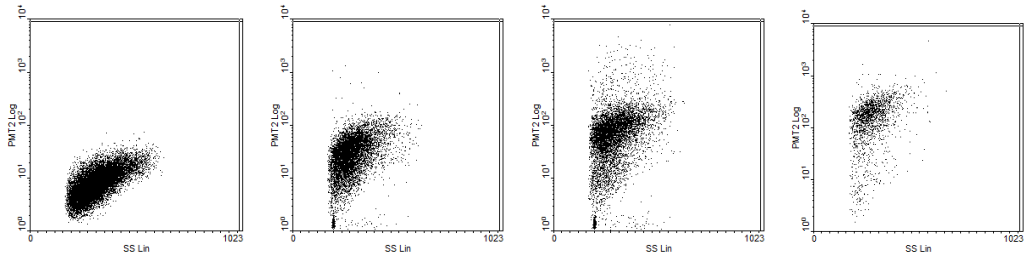


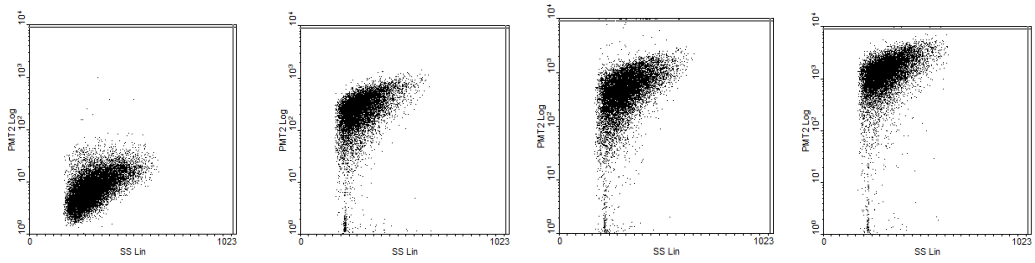
Fig 4-21: The MFI ± SDM readings for the uptake of different concentrations of nDer p 1 and rDer p 1 by immature DCs compared to the periodate treated preparations. Both n Der p 1 and rDer p 1 were treated with periodate for 30 mins and 1 hr, then their uptake was measured. The results shows a significant decrease in uptake of periodate treated preparations compared with the untreated ones.*** *P value*<0.001, all Der p 1 preparations were labelled with FITC. number of independent experiments=3

The results indicate a significant decrease in the uptake of both Der p 1 preparations after periodate treatment. The recombinant

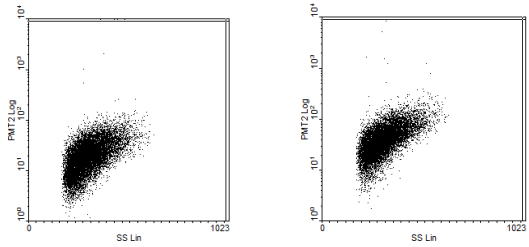
periodate treated preparation (1µg/ml) showed a 53% decrease in uptake after 30 mins of treatment compared to the untreated preparation of the same concentration; at 60 mins of periodate oxidation, the uptake decreased to 81.6%. The natural periodate treated sample showed a decrease of 58.7% after 30 mins of periodate treatment and 90% decrease in uptake after 60 mins of oxidation (Figs 4-20 & 4-21). Confocal imaging after 1 hr of periodate reaction showed minimal uptake by DCs for both Der p 1 preparations compared to untreated proteins (Fig 4-24 A & B). To highlight the importance of mannosylation in the uptake of proteins, a non-mannosylated cysteine protease, namely Staphopain B from *Staphylococcus aureus* (see chapter 2), was examined for its uptake by DCs compared to Der p 1 (natural and recombinant) and to the periodate treated preparations (Fig 4-22 & 4-23). The percentage uptake of natural Der p 1 compared to Staphopain B was higher by 64%. Confocal imaging for the uptake of Staphopain B showed a lower quantity of it inside DCs compared to the natural and recombinant preparations of Der p 1 (Fig 4-24 C).



Cells only nDerp1 0.25µg/ml nDerp1 0.5µg/ml nDer p 1 1.0µg/ml



nDerp1(1µg/ml)+periodate 1hr rDerp1 1µg/ml rDerp1 5µg/ml rDerp1 10µg/ml



rDerp1(1µg/ml)+ +period 1hr Staphopain B

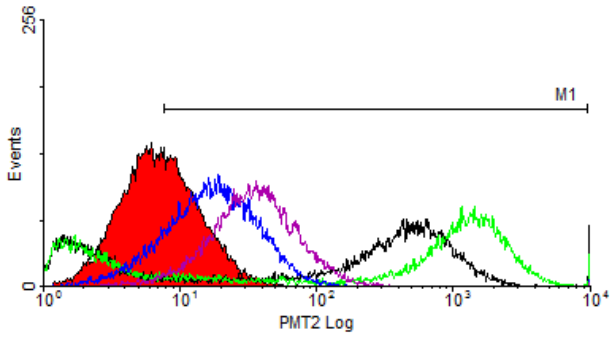


Fig 4-22: Representative dot plots of immature DC populations expressed as a histogram and as cell population by the WIN MDI

2.9 program after the uptake of different rDer p1 preparations and Staphopain B. Red: cells only, black: rDer p 1 0.5 µg/ml, green: r Der p 1 1.0 µg/ml, blue: periodate treated rDer p 1 1.0 µg/ml for 1 hr, purple: Staphopain B 1.0 µg/ml. All Der p 1 preparations were labelled with FITC. Staphopain B was labelled with FITC.

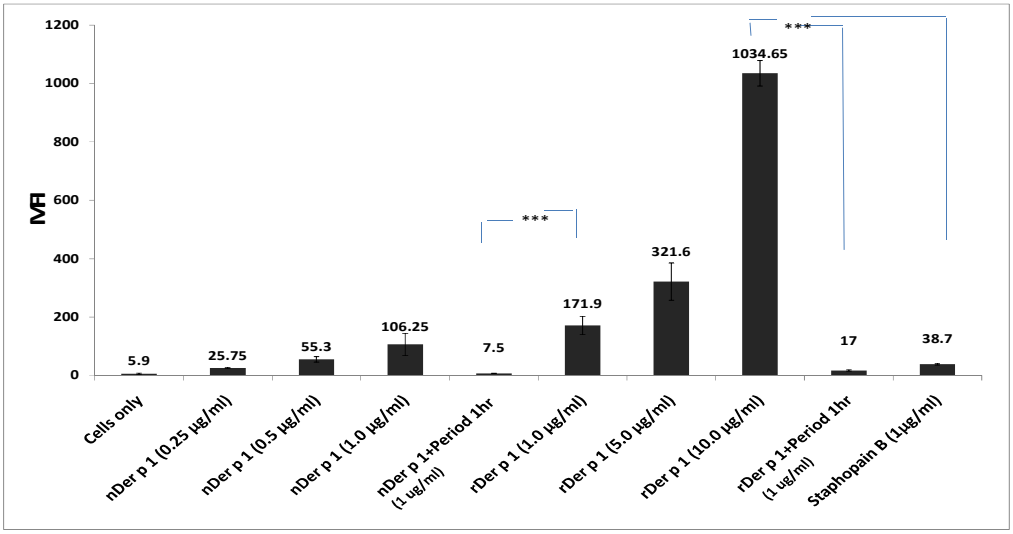
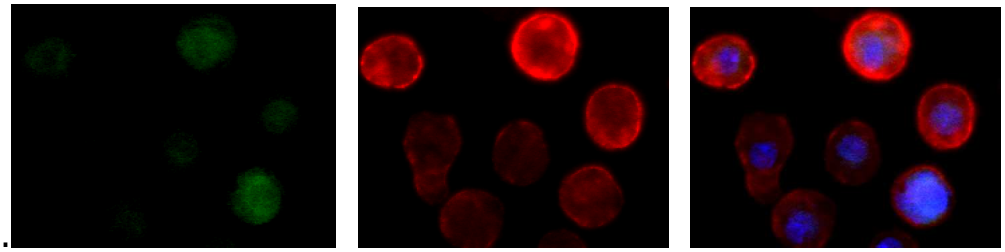


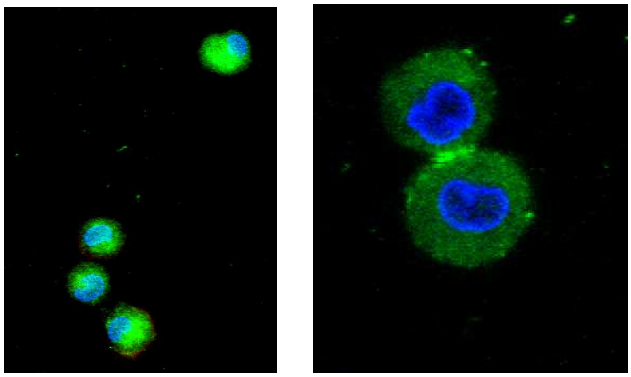
Fig 4-23: The MFI± SDM readings for the uptake of different concentrations of nDer p 1 and rDer p 1 by immature DCs compared to the periodate treated preparations and Staphopain B uptake. The results shows a decrease in uptake upon treating nDer p 1 and rDer p 1 with periodate.*** *P value*<0.001. All Der p 1 preparations were labelled with FITC. Staphopain B is also Labelled with FITC. number of independent experiments=3

To examine if mannan is the only inhibitor of Der p 1 uptake of allergens we examined the effect of galactose on uptake, DCs were incubated with a galactose polymer (200 µg/ml) and mannan (200 µg/ml) for 20 mins at 37°C and the uptake of Der p 1 was measured (Fig 4-25)4. The results indicate that galactose has no significant effect on the uptake of Der p 1 by DCs. At lower concentrations of galactose and mannan (50 µg/ml), mannan inhibited the uptake of Der p 1 while galactose did not (Fig 4-26)

A.

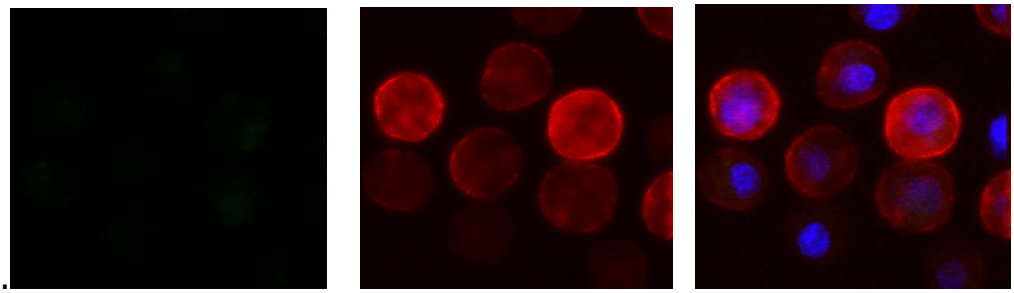


Periodate treated rDer p 1 MR Merged image

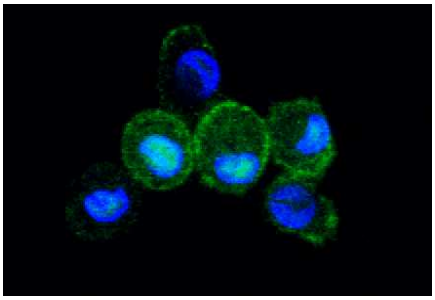


Control of rDer p 1 uptake(Green) with no periodate treatment

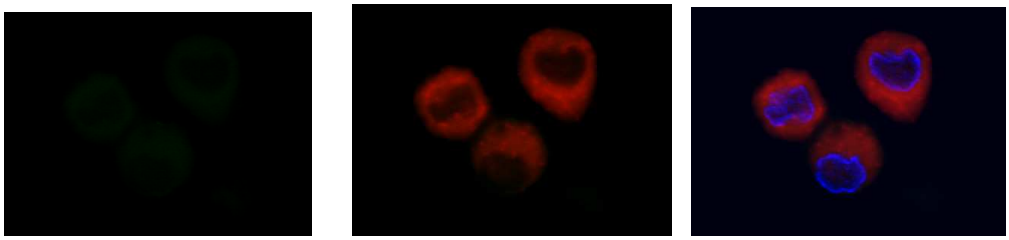
B.



Periodate treated nDer p 1 MR Merged image



Control of nDer p 1 uptake (Green) with no periodate treatment

C. 

Staphopain B MR Merged image

Fig 4-24: Confocal images showing the uptake of rDer p 1 and nDer p 1 when treated with sodium periodate for 1hr. The recombinant and natural Der p 1 concentration used is 1 µg/ml. Staphopain B was also used at a concentration of 1 µg/ml. The images show minimal uptake of periodate treated samples. Image C also shows reduced uptake of Staphopain B compared to the uptake of nDer p 1 allergen. A. Green: rDer p1 stained with FITC, red: MR stained with PE, blue: nucleus stained with DAPI. B. Green: nDer p 1 stained with Cy5, red: MR stained with PE, blue: nucleus stained with DAPI. C. Green: Staphopain B stained with FITC, red: MR stained with PE, blue: nucleus stained with DAPI, number of independent experiments=2.

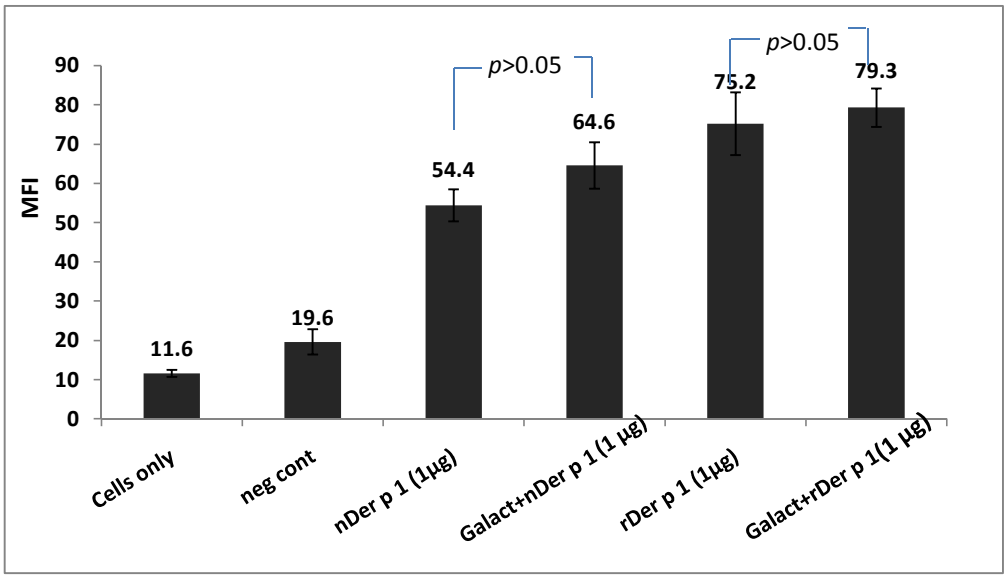


Fig 4-25: The MFI ± SDM readings for the uptake of different concentrations of nDer p 1 and rDer p 1 (1 µg/ml) by immature DCs compared to their uptake when adding galactose at 200 µg/ml. Adding galactose did not affect the uptake of nDer p 1 and rDer p 1. P value> 0.05 is considered non-significant. number of independent experiments=3.

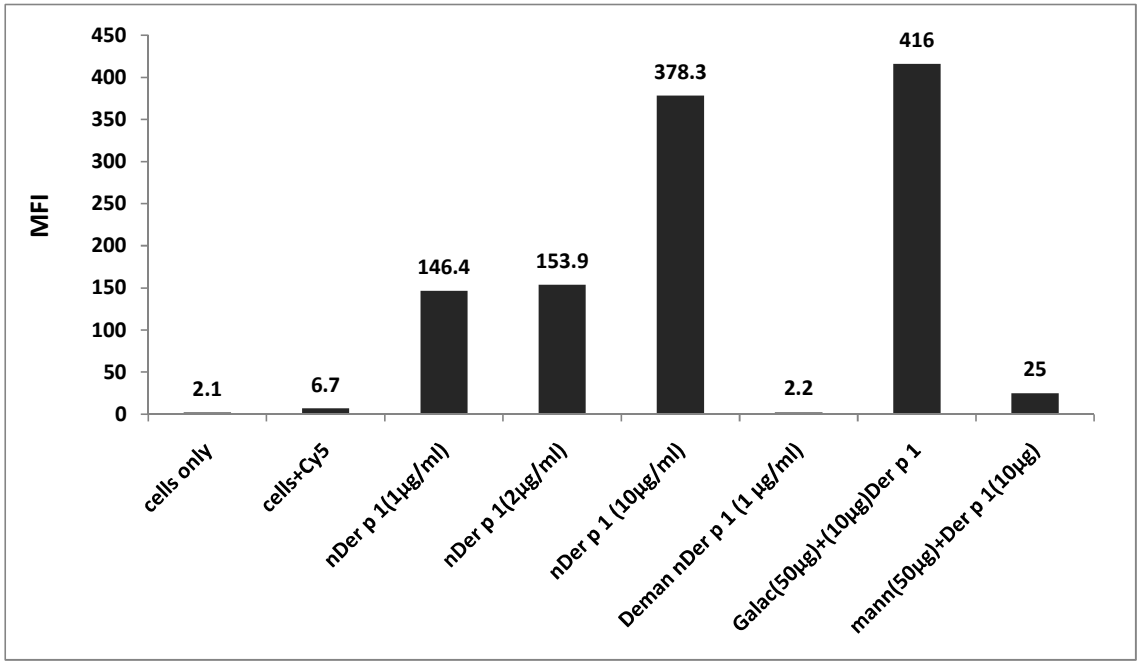
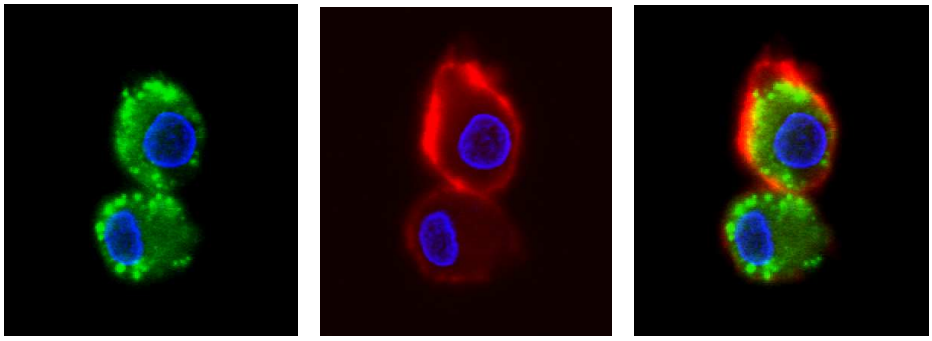


Fig 4-26: The MFI ± SDM readings for the uptake of different concentrations of nDer p 1 by immature DCs compared to uptake when adding galactose and mannan at 50 µg/ml. adding mannan at a concentration of 50µg/ml reduced the uptake of nDer p1 while adding galactose at the same concentration of 50µg/ml did not affect the uptake of nDer p 1, number of independent experiments=1.



nDer p 1 MR merged image

Fig 4-27: Confocal images showing the uptake of nDer p 1 (1µg/ml) by immature DCs when adding galactose at 200 µg/ml. The image shows that there is an uptake for nDer p1 after saturating the DCs with galactose, number of independent experiments=3. (Compare to mannan inhibition of uptake in Fig 4-18B)

4.3.4 MR binding of the different glycoforms of Der p 1:

The different glycoform preparations of Der p 1 were shown in chapter 2 & 4 to contain different carbohydrate contents. It was, therefore, of interest to know if the binding to MR subfragment CTLD 4-7, the C-type lectin carbohydrate recognition domain, will differ according to the carbohydrate content of the glycoforms. Mannan and galactose were used as positive and negative controls, respectively. Results in Fig 4-28 show a significant decrease in binding to MR (>55%) when Der p 1 allergen is deglycosylated with periodate for 60 mins. The same is noticed when treating hypermannosylated rDer p 1 with periodate for 60 mins as the decrease in binding after

deglycosylation reached 42.5%. It is clear that the binding of the recombinant preparation of Der p 1 to MR is much stronger than that of natural Der p 1 which is expected as rDer p 1 has more mannan than natural Der p 1 (shown in chapter 2). Another recombinant glycoform used here is D Der p 1, which is an Aglycosylated recombinant preparation that was produced through a mutation in rDer p 1 glycosylation site. This preparation binds MR less than the recombinant Der p 1 by 27%. Fig 4-29 shows that the periodate-treated glycoforms still bind 5H8 anti-Der p 1 antibody, indicating that periodate treatment did not change the conformation of the protein, but just altered the sugar content.

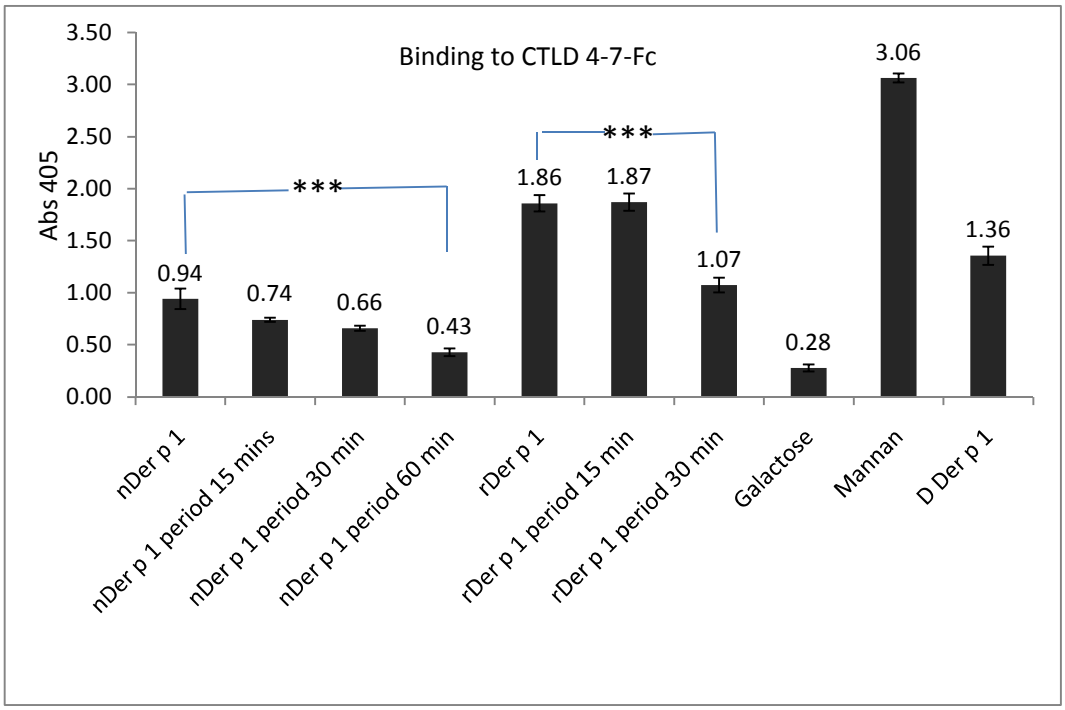


Fig 4-28: Binding of MR C-type lectin-like carbohydrate recognition domains 4-7 with different glycoforms of Der p 1: Der p 1, rDer p 1 and D Der p 1 (recombinant deglycosylated Der p 1 with mutation N52Q) (concentrations at 2µg/ml). ****P* value < 0.001, number of independent experiments=3.

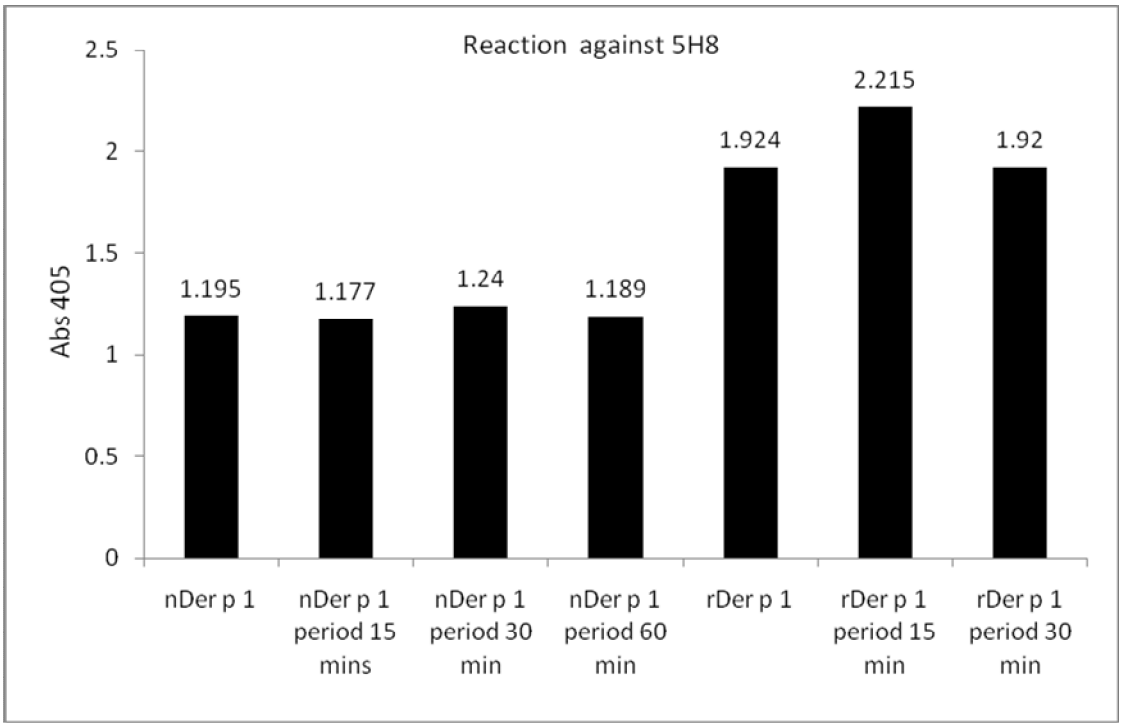


Fig 4-29: Binding of anti-Der p1 5H8 antibody to different glycoforms of Der p 1 (2µg/ml), number of independent experiments=2.

4.3.5 Differences in TSLP secretion induced by the different glycoforms of Der p 1:

The next step in showing that the glycan content of allergens could alter the outcome of Th1/Th2 immune responses is by measuring Th2 cytokine secretions after exposure to allergens. An initial example of this is TSLP secretions that are secreted by epithelial cells in humans and mice and are thought to induce Th2 immune responses. We incubated the different glycoforms of Der p 1 with human epithelial cells for 24 hrs, then TSLP was

measured by a human TSLP development kit. Results in Fig 4-30 & 4-31 show a significant increase in TSLP secretion by human epithelial cells when challenged by Der p 1, and this increase in TSLP becomes less pronounced when periodate deglycosylated Der p 1 was used. However, we were unable to reproduce this pattern with the recombinant preparation of Der p 1. The increase in the secretion of TSLP by human epithelial cells upon stimulation with Der p 1 may therefore lead to a Th2 biased immune response.

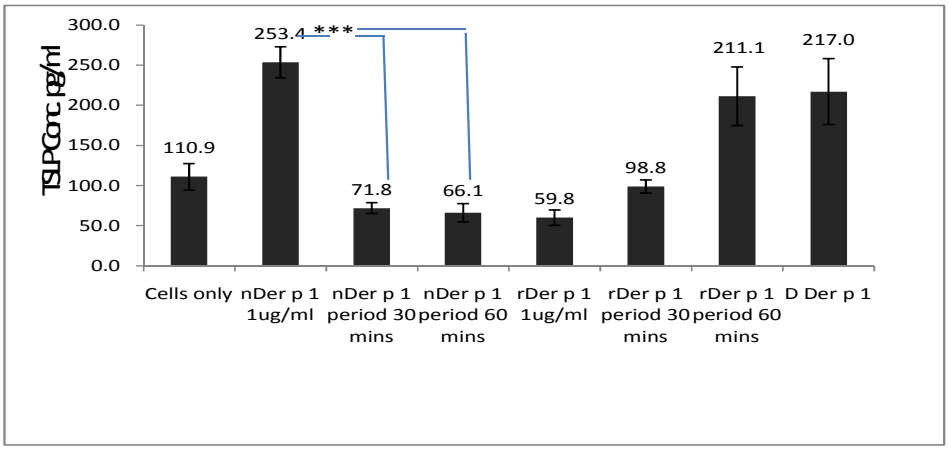


Fig 4-30: Differences in TSLP secretion in human BEAS-2B epithelial cells after 24 hrs stimulation with different glycoforms of Der p 1. Concentration of Der p 1 used was 1 μg/ml. ****P* value < 0.001, number of independent experiments=3.

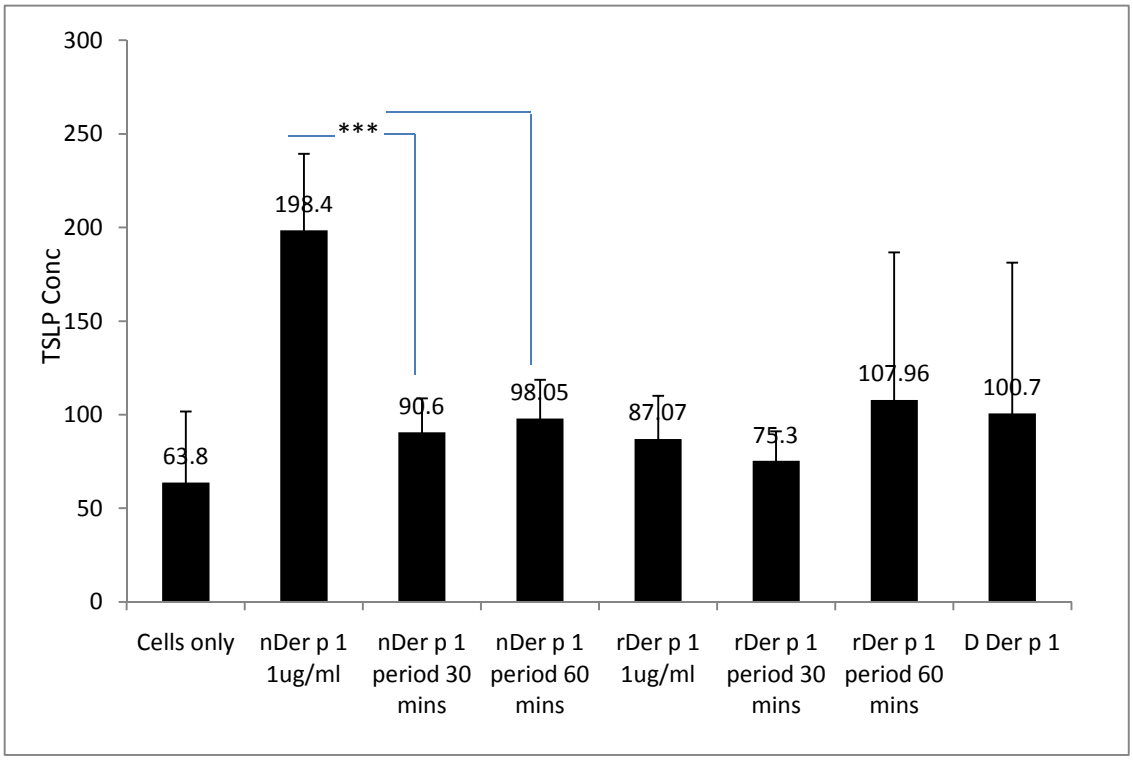
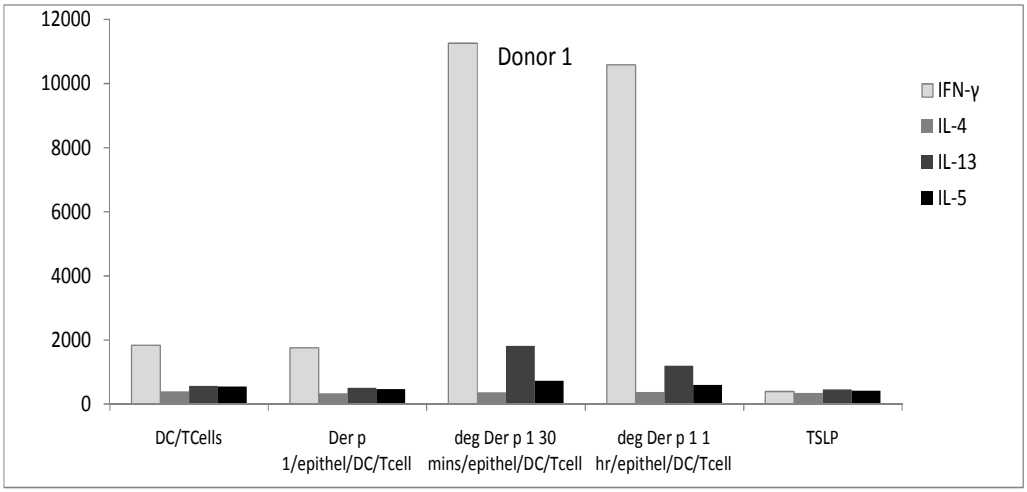


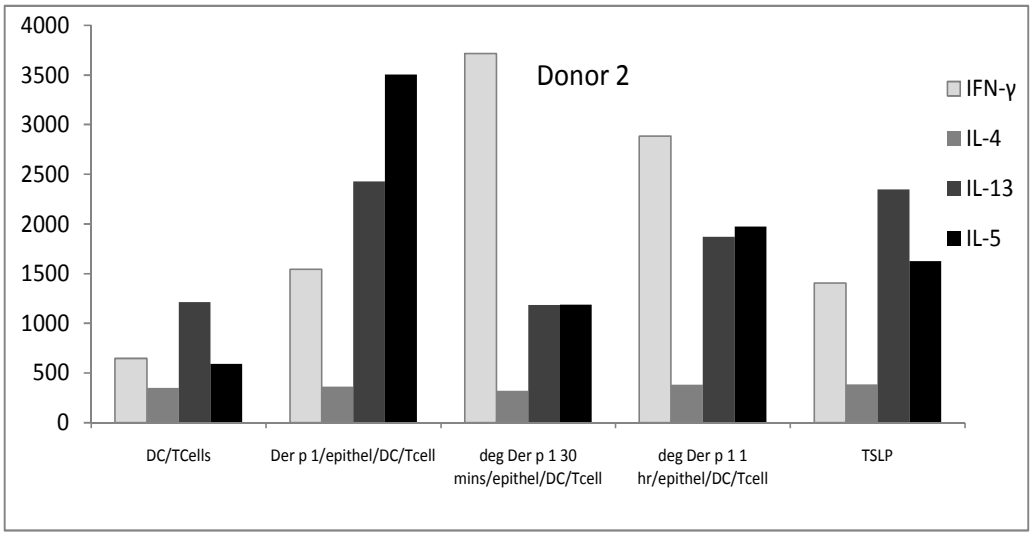
Fig 4-31: Differences in TSLP secretion in human BEAS-2B epithelial cells when co-cultured for 48 hrs with human Lung bronchial fibroblasts cells and then stimulated with different glycoforms of Der p 1 for 24 hrs. Concentration of Der p 1 used was 1 µg/ml. ****P* value < 0.001, number of independent experiments=3.

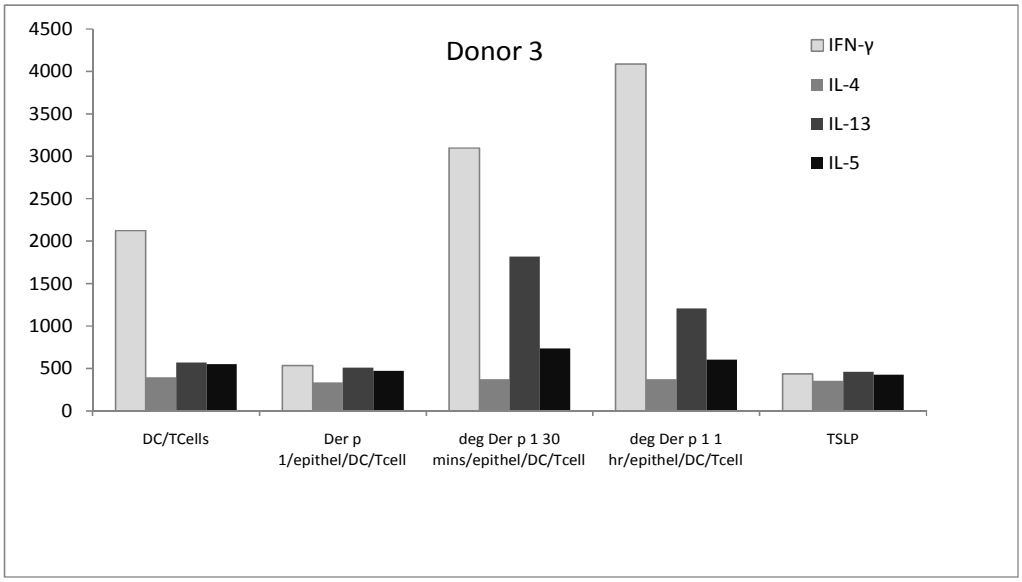
4.3.6 Differences in T cell cytokine secretion induced by different glycoforms of Der p 1:

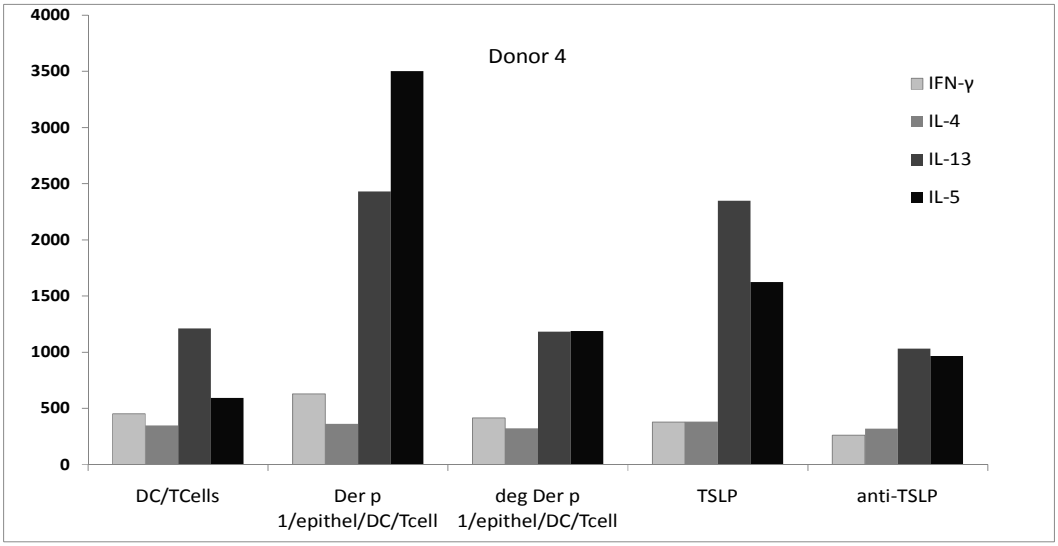
To investigate the impact of Der p 1-induced TSLP secretion in human epithelial cell media on T cell cytokine profile, TSLP-conditioned human DCs were co-cultured with autologos T cells and IL-4, 5, 13 and IFN-γ cytokine secretions were measured.

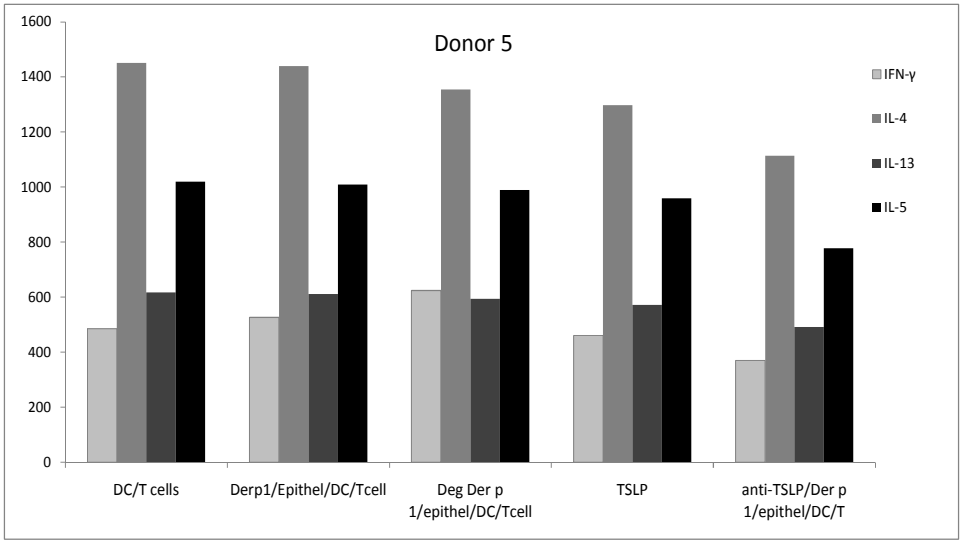
Results shown in Fig 4-32 is for atopic blood and the data for donors 2 and 4 indicate a high IL-5 and IL-13 on average upon induction with high TSLP enriched media (produced via natural Der p 1 stimulation of epithelia) compared to low TSLP induction produced though stimulating the epithelial cells with deglycosylated Der p 1 preparations. Donors 1, 2 and 3 however showed a very high IFN-γ when low TSLP induction was used upon stimulation with periodate deglycosylated Der p 1. This may indicate a shift towards a Th1 immune response when deglycosylating Der p 1. Upon introducing an antibody against TSLP in donors 4-6 to the epithelial media that is stimulated with native Der p 1, cytokine secretion decreases on average, which may suggest that TSLP cytokine is responsible for increasing IL-5 and IL-13 cytokine secretion that eventually lead to Th2 bias immune response.


Donor 1
12000
10000
8000
6000
4000
2000
0
IFN-γ
IL-4
IL-13
IL-5
DC/TCells
Der p 1/epithel/DC/Tcell
deg Der p 1 30 mins/epithel/DC/Tcell
deg Der p 1 1 hr/epithel/DC/Tcell
TSLP


Donor 2
4000
3500
3000
2500
2000
1500
1000
500
0
IFN-γ
IL-4
IL-13
IL-5
DC/TCells
Der p 1/epithel/DC/Tcell
deg Der p 1 30 mins/epithel/DC/Tcell
deg Der p 1 1 hr/epithel/DC/Tcell
TSLP


Donor 3
IFN-γ
IL-4
IL-13
IL-5
4500
4000
3500
3000
2500
2000
1500
1000
500
0
DC/TCells
Der p 1/epithel/DC/Tcell
deg Der p 1 30 mins/epithel/DC/Tcell
deg Der p 1 1 hr/epithel/DC/Tcell
TSLP


Donor 4
IFN-γ
IL-4
IL-13
IL-5
4000
3500
3000
2500
2000
1500
1000
500
0
DC/TCells
Der p 1/epithel/DC/Tcell
deg Der p 1/epithel/DC/Tcell
TSLP
anti-TSLP


Donor 5
1600
1400
1200
1000
800
600
400
200
0
IFN-γ
IL-4
IL-13
IL-5
DC/T cells
Derp1/Epithel/DC/Tcell
Deg Der p 1/epithel/DC/Tcell
TSLP
anti-TSLP/Der p 1/epithel/DC/T

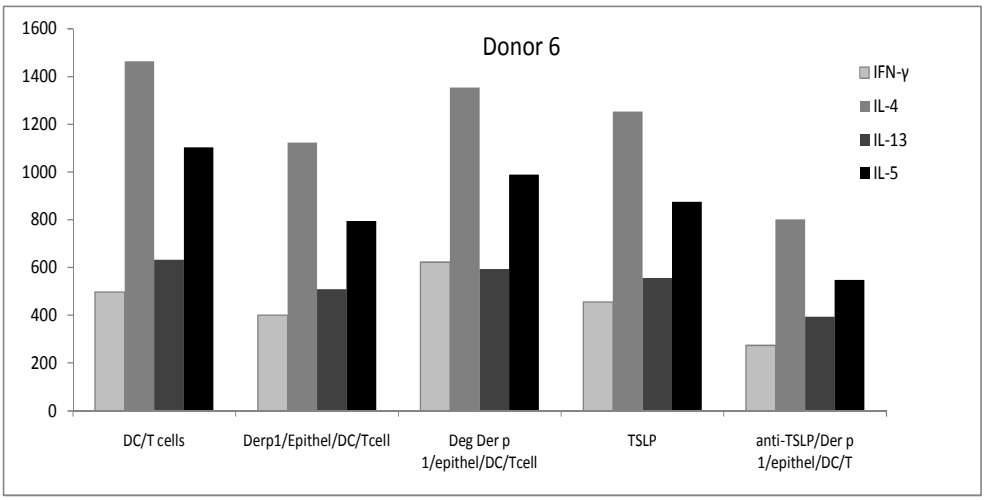

Donor 6
1600
1400
1200
1000
800
600
400
200
0
IFN-γ
IL-4
IL-13
IL-5
DC/T cells
Derp1/Epithel/DC/Tcell
Deg Der p 1/epithel/DC/Tcell
TSLP
anti-TSLP/Der p 1/epithel/DC/T

Fig 4-32 : T cell cytokine secretion upon stimulating DCs with different TSLP containing media and co-culturing them with T cells *in vitro* (atopic human blood). In donors 1, 2 and 3 there is an increase in IFN-γ secretion upon stimulation with deglycosylated preparations of Der p 1 compared to lower IFN-γ secretion when native Der p 1 is used. There is an overall decrease in IL-5 and 13 secretions when using deglycosylated Der p 1 preparations in donors 2 and 4. Donors 5 and 6 had no change in cytokine secretions upon glycoforms stimulation, number of independrnt experiments=6.

4.4 Discussion:

This work is an attempt to gain a better understanding of the role of glycosylation in Der p 1 recognition by the innate immune system. The recombinant Der p 1 preparation used in this study was produced in *Pichia pastoris* and as such it is hypermannosylated (Cereghino et al., 2002, Cregg, 2007). It is therefore interesting that this highly mannoasylated preparation was taken up more readily by DCs than natural Der p 1, and this underlines the importance of sugars in allergen recognition by MR and presumably by DC-SIGN (Royer et al., 2010, Taylor et al., 2005, Geijtenbeek et al., 2000, Hsu SC, 2010). This was further confirmed by the demonstration that the

hypermannosylated recombinant Der p 1 preparation was able to inhibit, the uptake of natural Der p 1 by DCs.

Mannan itself inhibited the uptake of both recombinant and natural Der p 1 by up to 88% and 83% respectively, which is expected given that mannan is the natural ligand for MR. Moreover, the mannose receptor has been shown to play a key role in the uptake of diverse allergens by human DCs and in the bias towards the Th2 phenotype (Royer et al., 2010, Taylor et al., 2005, Deslee et al., 2002).

The above data were further corroborated by demannosylating rDer p 1 via a combination of enzymes and chemical deglycoslatiom resulting in a preparation exhibiting minimal uptake by DCs. This was best exemplified by sodium metaperiodate treatment of rDer p 1. Sodium metaperiodate treatment does not affect the protein under mild conditions, and in one study it was shown that at 10 mM could lead to structural alterations in carbohydrates without any significant effect on proteins in *Schistosoma mansoni* Egg Antigens (Okano et al., 1999). Periodate was also used to destroy carbohydrates on Cry j 1, the major allergen of Japanese cedar pollen, and it was shown that after periodate oxidation Cry j 1-specific CD4+ T cell proliferation decreased significantly and there was also

significantly less IL-4 and IL-5 secretion in comparison with the control antigen (Mitsuhiro et al., 2001). Consequently, those authors suggested a role for carbohydrates on Cry j 1 in promoting Th2 immune responses *in vitro.*

The confocal images provided an insight into the uptake of different Der p 1 preparations by DCs. It became clear that the internalization of Der p 1 is initiated by MR on immature DCs. Confocal imaging also showed that recombinant Der p 1 uptake is immediate, starting at 5 mins of incubation and that a higher quantity accumulates inside the DC compared to natural allergen. Recombinant and natural Der p 1 both co-localised with LAMP-2, a lysosomal marker, suggesting a common fate for these preparations inside the DC. There is a possibility that the apparent differences in staining with rDerp1 and nDerp1 could be due to differences in labelling efficiencies with the fluorochrome preparations and/or other differences in the Derp1 preparations (unrelated to glycosylation levels). However, the confocal results are consistent with the flow cytometry studies that indicate higher uptake of rDerp1 when using the same fluorochromes for both rDerp1 and nDerp1 preparations, and also with ELISA studies of binding to MR using unlabelled Derp1 preparations.

The uptake of the non-allergenic protein Staphopain B, a cysteine protease from *Staphylococcus aureus*, was significantly lower than that of nDer p 1 (also a cysteine protease), as shown by confocal imaging and FACS. This was expected as this cysteine protease is not mannosylated (see chapter 2).

This chapter also showed that the different glycoforms of Der p1 bind less to the lectin-like carbohydrate recognition domains 4-7 of MR compared to the native one. This is expected as these glycoforms proved to have less mannan than the natural preparation. The fact that they lost their sugar content did not affect the whole protein structure as they still bind to anti-Der p 1 5H8. This indicates that they have the same conformation as natural allergens.

Der p 1 is an allergen that induces Th2 immune response which provokes high IL-4, IL-5 and IL-13 cytokine secretions by T cells and increase secretion of TSLP by epithelial cells. This finding seems to change when challenging human epithelial cells with a deglycosylated preparation of Der p 1. The TSLP secretion is reduced when culturing the epithelial cells with different

deglycosylated forms of Der p 1 compared to the natural preparations.

Upon stimulating DCs with the different concentrations of TSLP containing media (produced by Der p 1 glycoforms stimulation of epithelial cells) and co-culturing them with T cells *in vitro,* it seems that low concentrations of TSLP induced the production of lower IL-4,5 and 13 by T cells on average in some donors and a high concentrations of TSLP stimulated T cells to produce higher IL-4, 5 and 13. In some experiments low TSLP concentrations produced through deglycosylating Der p 1 induced higher IFN-γ and lower IL-5 and IL-13 indicating a shift towards Th1 immune response when using deglycosylated Der p 1 allergen in some donors. Using an antibody against TSLP in the same media showed that the cytokine secretions by T cells decreased on average which suggests that TSLP cytokine is responsible for increasing IL-5 and IL-13 cytokine secretion.

In conclusion, glycosylation is clearly a key feature of many allergens (as described in chapter 2), and mannan seems to be the dominant sugar moiety associated with allergens. It is therefore not surprising that MR plays such a significant role in

the recognition and internalisation of many diverse allergens (Royer et al, 2010). This chapter investigated the nature of glycoallergen recognition by DCs through studying the uptake of labelled hyper and hypo-glycosylated preparations of Der p 1. Data generated provided an insight into the effect of mannosylation on Der p 1 recognition and uptake by DCs.

CHAPTER FIVE: General discussion

The role of allergen glycosylation in allergy has not been fully elucidated yet. Many allergens are naturally glycosylated and are known to contain carbohydrate determinants of xylose and fucose, and this raises the possibility that the specific glycosyl groups may contribute to allergens immunogenicity and allergenicity. The main aim of this project was to clarify the role of protein glycosylation in allergens' recognition by the innate immune system. This was assessed through investigating the pattern of glycosylation in major allergens like Der p 1, Fel d 1, Ara h 1, Ber e 1, Der p 2, Bla g 2, Can f 1, Bromelain and Papain by using specified lectins like GNA, which helped defining N-glycosylation. We also used SNA, MAA, PNA and DSA that recognised specific O-glycosylation. These experiments showed that all major allergens are indeed glycosylated and this represented a major first step towards demonstrating a link between glycosylation and allergen recognition by the innate immune system. Most of the previous research carried out on allergen glycosylation reported the presence of xylose and fucose (Altmann, 2007, Chunsheng et al., 2008). We, however, found that the main dominant sugars on allergens are 1,2 1,3 and 1,6 mannose,

as detected by GNA lectin, and this was present in high quantities in allergens like Ara h 1, Bla g 2 and Papain compared to non-allergens. We have also shown that Der p 1 and Der p 2 possess 1,3 fucose in their natural forms, thus concluding that Der p1 and Der p 2 have part of the CCDs which are epitope structures for IgE. This was not reported before and therefore aided our attempts to have a better understanding of allergen N and O-glycosylation patterns as a whole. We were also able to describe differences in glycosylation patterns between allergens and non-allergens, which were mainly due to variation in mannosylation and fucosylation.

Comparative carbohydrate analysis between allergens and non-allergens from the cysteine protease family, which included Der p 1, Papain and Bromelain as allergens and Staphopain B, Calpain and Cysteine Protease B (CPB) as non-allergens, indicated that the non-allergens Staphopain B and Calpain do not react with GNA, and thus are not mannosylated like allergens. Cysteine protease B showed a very weak reaction with GNA, again indicating that it has only a minimal amount of mannose moities. Staphopain B also did not react with any of the other lectins, indicating that it does not have any O-glycosylation.

We were able to predict N-glycosylation structures on major allergens when combining the data we obtained from lectin reactivity with the data already present in the literature. Der p 1 for example reacted with GNA and 1,3 fucose and the literature reported that xylose is present in Der p 1. Thus, its N-glycosylation pattern was predicted with the help of symbolic representation suggested by the Consortium of Functional Glycomics. Similar speculations were done for several major allergens like Ara h 1, Bla g 2, Can f 1 and Fel d 1 (Fig 2-10). A pattern for the difference in quantity of mannosylation emerged between allergens and non-allergens, which can be concluded by saying that non-allergens possess a very low quantity of mannose and fucose 1, 3.

O-glycosylation in allergens was also studied thoroughly through SNA, MAA, PNA and DSA lectin reactivities, giving a better understanding of the whole glycan structure in allergens.

The second step was to study if any changes in the sugar content of allergens would affect their recognition by the immune system. This was facilitated through optimising different methods to produce glycoforms of the same allergen

and assess their recognition by cells of the innate immune system.

In an attempt to express a recombinant hyperglycosylated form of Der p 1 allergen in *Pichia pastoris*, which is known to produce hypermannosylated proteins, Der p 1 cDNA was successfully amplified from a phage library, sequenced, inserted into a vector, transformed to TOP 10 cells for amplification and concentration. The vector was then purified, concentrated, transformed to *Pichia* yeast X33 competent cells and expressed. In order to produce deglycosylated forms of Der p 1 allergen, mutations to possible glycosylation sites (N16, N82, N132, N195) in the proDer p 1 amplicon were done and sequenced then expressed in *Pichia*. Again these mutants were successfully inserted into a vector, transformed to TOP 10 cells for amplification. The vector was then purified, concentrated, transformed to *Pichia* competent cells and expressed. We were able to show that Der p1 and its deglycosylated mutants were expressed in *Pichia* as they reacted to 5H8 anti Der p 1 antibody. However, the expression of these amplicons in yeast seemed to be affected by culture proteases resulting in degradation, thus other methods with more consistent results were optimised to

produce these glycoforms, which are discussed in chapter four.

By optimising enzymatic and chemical deglycosylation methods, we were able to produce different glycoforms of Der p 1 which proved to possess less mannan than the natural allergen when reacting with GNA. A commercially available recombinant preparation was used as a hyperglycosylated allergen for Der p 1 and was shown to be highly mannosylated compared to the natural form. All these glycoforms reacted with anti-Der p 1 5H8 antibody, which confirms that the allergen conformation and epitope structure remained intact. These glycoforms were useful tools in addressing the glycoallergen recognition pattern by looking directly at the uptake of fluorochrome labelled hyper- and hypo-glycosylated preparations of the same allergen by DCs via confocal microscopy, ELISA and FACS studies. Comparative analysis of the recombinant hypermannosylated preparation of Der p 1 and its natural counterpart, which possess less mannan, showed that the recombinant form was taken up more readily by DCs at 37°C and at 4°C, as shown by FACS and confocal imaging (Figs 4-1, 4-2). The recombinant preparation was also able to inhibit the uptake of natural Der p 1 by DCs in a concentration dependent manner (Fig 4-4).

Furthermore, the uptake of the deglycosylated recombinant preparation of Der p 1 (N52Q) was less than that of the recombinant glycosylated preparation (Fig 4-3). This strongly suggests a role for glycosylation in uptake by immature DCs. When using the periodate deglycosylated Der p 1 and enzymatically deglycosylated Der p 1, they were not internalised in the same manner by DCs as their glycosylated counterpart and both exhibited minimal uptake by DCs as shown by FACS and confocal analysis (Figs 4-16, 4-21). The deglycosylated preparations of the allergen had minimal mannan as shown by western blotting against anti-mannose GNA (Fig 4-19), suggesting a key role for mannan in allergen uptake by DCs. A completely demannosylated Der p 1 allergen failed to be internalised by DCs as shown by confocal and FACS studies in chapter four (Figs 4-21, 4-23). The uptake of the non-allergen Staphopain B by DCs was minimal compared to allergens, even though this protein is a cysteine protease just like Der p 1. This may be due to the fact that this protein is not mannosylated as shown in chapter 2. Results also indicate that galactose has no significant effect on the uptake of Der p 1 by DCs (Fig 4-26).

The confocal images showed that the internalization of Der p 1 is initiated by MR on immature DCs. Recombinant and

natural Der p 1 both co-localised with MR, DC-SIGN and LAMP-2, albeit with different kinetics, which indicates a common fate for these preparations inside the DC. We also showed by ELISA experiments that these glycoforms bind to the MR subfragment CTLD 4-7, the C-type lectin carbohydrate recognition domain, and this is consistent with their carbohydrate content. Results show a significant decrease in binding to MR (>55%) when the Der p 1 allergen is deglycosylated with periodate for 60 mins. The same is noticed when treating hypermannosylated rDer p 1 with periodate for 60 mins, as the decrease in binding after deglycosylation reached 42.5%. It is clear that the binding of the recombinant preparation of Der p 1 to MR is much stronger than that of natural Der p 1 which is expected as rDer p 1 has more mannan than natural Der p 1 (shown in chapter 2).

Dendritic cells are specialised antigen presenting cells that direct the immune systems towards development of Th1 or Th2 immune responses. DCs express C-type lectin receptors that serve as antigen receptors allowing internalization and antigen presentation and also serve as signalling molecules. Several groups have demonstrated that glycan modification of antigens can change its presentation (Adams et al., 2008,

Aarnoudse et al., 2008, Mitsuhiro et al., 2001). Glycan modified ovalbumin having lewis oligosaccharides enhanced both CD4+ and CD8+ T cell responses (Singh et al., 2009, Wang et al., 2007). Others demonstrated that high mannose modified melanoma antigen enhanced antigen presentation to CD4+ T cells (Aarnoudse et al., 2008). Potentially lower or slower uptake upon deglycosylation of allergens could lead to change in the outcome of the innate and adaptive immune responses. This was presented in literature when Carbohydrates on Cry j 1 were destroyed by periodate-oxidation which resulted in significant decrease in T cells proliferation and a decrease in IL-4,5 production (Mitsuhiro et al., 2001).

This work addressed the nature of glycoallergen recognition through looking at the recognition and uptake of hyper- and hypo-glycosylated preparations of Der p 1. As allergens influence both innate and adaptive immune responses and favour Th2 immunity, it would be interesting to know if deglycosylated allergens stimulate the immune system differently. There is a substantial body of evidence showing that the encounter of allergens with epithelial cells induce TSLP secretion, which activates DCs allowing them to prime naive T cells for the production of pro-inflammatory cytokines

(Roggen et al., 2006). TSLP has been shown to induce IL-4, 5 and 13 driven allergic inflammation and upregulates MHC I and II co-stimulatory molecules CD40, CD80, CD83 and CD86 (Wang and Xing, 2008, Wang and Liu, 2009, Friend SL, 1994, Ray et al., 1996, Tanaka et al., 2009, Miyata et al., 2009, Soumelis et al., 2002).

In this work, some experiments were done to show the effect of Der p 1 and Der p 1 glycoforms on TSLP secretion *in vitro*. We showed that there is a significant increase in the secretion of TSLP by epithelial cells when encountering Der p 1 compared to the control, and that TSLP secretion decreases significantly when epithelial cells are challenged with deglycosylated preparations of the same allergen. This observation should now pave the way for looking at the differential expression of Th1/Th2 cytokines following exposure of epithelial cells to different allergen glycoforms and co-culturing TSLP-conditioned DCs with T cells

More work is needed to have a better understanding of the effect of carbohydrates on adaptive immunity and if differences in glycosylation patterns could explain why certain proteins act as allergens. This could be facilitated by producing different glycoforms of other major allergens

through chemical deglycosylation or recombinant expression and examining their recognition by APCs and their downstream effects on T cells.

There is considerable interest in defining why allergens are allergens. This work has clearly progressed the notion that allergenicity is possibly linked to the glycosylation pattern of allergens. It is therefore reasonable to conclude that mannan seems to be the dominant sugar moiety associated with allergens, and this is consistent with MR being the main receptor involved in allergen recognition and uptake by DCs.

CHAPTER SIX: Bibliography

AARNOUDSE, C. A., BAX, M., SÁNCHEZ-HERNÁNDEZ, M., GARCÍA-VALLEJO, J. J. & VAN KOOYK, Y. (2008). Glycan modification of the tumor antigen gp100 targets DC-SIGN to enhance dendritic cell induced antigen presentation to T cells. International Journal of Cancer, 122, 839-846.

ADAMS, E. W., RATNER, D. M., SEEBERGER, P. H. & HACOHEN, N. (2008). Carbohydrate-Mediated Targeting of Antigen to Dendritic Cells Leads to Enhanced Presentation of Antigen to T Cells. ChemBioChem, 9, 294-303.

ADNAN, C., SIMON, C. O. T., HELEN, C. F., MARTIN, D. C. & ASHLEY, W. (1996). Exposure to house dust mite allergens and the clinical activity of asthma. The Journal of Allergy and Clinical Immunology, 98, 64-72.

AKDIS, C. A. (2006). Allergy and hypersensitivity: mechanisms of allergic disease. Current Opinion in Immunology, 18, 718-26.

ALTMANN, F. (2007). The Role of Protein Glycosylation in Allergy. International Archives of Allergy and Immunology, 142, 99-115.

ANDREW, H. L. (2004). Something old, something new: Indoor endotoxin, allergens and asthma. Paediatric Respiratory Reviews, 5, S65-S71.

ANNE, I., SVEN, B., STEPHAN, S., ZOE, W., RYOJI, N., ANNE, W., YASUHIKO, Y., HIROSHI, Y., THOMAS, H., CHRISTIAN, K., ULRICH, K., STEFAN, V. & MASAKO, T. (2010). Glycation of a food allergen by the Maillard reaction enhances its T-cell immunogenicity: Role of macrophage scavenger receptor class A type I and II. The Journal of Allergy and Clinical Immunology, 125, 175-183.e11.

AOKI, R., SAITO, A., AZAKAMI, H. & KATO, A. (2010). Effects of Various Saccharides on the Masking of Epitope Sites and Uptake in the Gut of Cedar Allergen Cry j 1−Saccharide Conjugates by a Naturally Occurring Maillard Reaction. Journal of Agricultural and Food Chemistry, 58, 7986-7990.

ASARCH, A., BARAK, O., LOO, D. S. & GOTTLIEB, A. B. (2008). Th17 cells: A new paradigm for cutaneous

inflammation. Journal of Dermatological Treatment, 99999, 1 - 8.

BARDOR, M., FAVEEUW, C., FITCHETTE, A.-C., GILBERT, D., GALAS, L., TROTTEIN, F., FAYE, L. & LEROUGE, P. (2003). Immunoreactivity in mammals of two typical plant glyco-epitopes, core {alpha}(1,3)-fucose and core xylose.

BEST, E. A., STEDMAN, K. E., BOZIC, C. M., HUNTER, S. W., VAILES, L., CHAPMAN, M. D., MCCALL, C. A. & MCDERMOTT, M. J. (2000). A Recombinant Group 1 House Dust Mite Allergen, rDer f 1, with Biological Activities Similar to Those of the Native Allergen. Protein Expression and Purification, 20, 462-471.

BETTELLI, E., KORN, T. & KUCHROO, V. K. (2007). Th17: the third member of the effector T cell trilogy. Current Opinion in Immunology, 19, 652-657.

BIRD, L. (2008). Allergy and Asthma: Calling all TH2 cells. Nat Rev Immunol, 8, 162-162.

BROOKS, F. J., BUTEL, S. J. & MORSE, A. S. (2004). Medical Microbiology, Lange.

BÜHLING, F., FENGLER, A., BRANDT, W., WELTE, T., ANSORGE, S. & NAGLER, D. (2002). Review: Novel Cysteine

Proteases of the Papain Family. Cellular Peptidases in Immune Functions and Diseases.

BURTIN, D., CHABRE, H., OLAGNIER, B., DIDIERLAURENT, A., COURET, M. N., COMEAU, D., WAMBRE, E., LAPARRA, H., VAN OVERTVELT, L., MONTANDON, F., BATARD, T., JONVAL, V., LORPHELIN, A., MERLE, C., BERROUET, C., PARRY, L., GOMORD, V., VAN REE, R. & MOINGEON, P. (2009). Production of native and modified recombinant Der p 1 molecules in tobacco plants. Clinical & Experimental Allergy, 39, 760-770.

CALABOZO, B., BARBER, D. & POLO, F. (2002). Studies on the carbohydrate moiety of Pla l 1 allergen. Identification of a major N-glycan and significance for the immunoglobulin E-binding activity.

CEREGHINO, G. P. L., CEREGHINO, J. L., ILGEN, C. & CREGG, J. M. (2002). Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris. Current Opinion in Biotechnology, 13, 329-332.

CEREGHINO, J. L. & CREGG, J. M. (2000). Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiology Reviews, 24, 45-66.

CHAPLIN, D. (2006). Overview of the human immune response. The Journal of allergy and clinical immunology, 117, S430-S435.

CHAPLIN, D. (2010). Overview of the immune response. The Journal of Allergy and Clinical Immunology, 125, S3-S23.

CHAPMAN, M. D., POMÉS, A., BREITENEDER, H. & FERREIRA, F. (2007). Nomenclature and structural biology of allergens. Journal of Allergy and Clinical Immunology, 119, 414-420.

CHAPMAN, M. D., SMITH, A. M., VAILES, L. D., ARRUDA, L. K., DHANARAJ, V. & POMÉS, A. (2000). Recombinant allergens for diagnosis and therapy of allergic disease. Journal of Allergy and Clinical Immunology, 106, 409-418.

CHARBONNIER, A.-S., HAMMAD, H., GOSSET, P., STEWART, G. A., ALKAN, S., TONNEL, A.-B. & PESTEL, J. (2003). Der p 1-pulsed myeloid and plasmacytoid dendritic cells from house dust mite-sensitized allergic patients dysregulate the T cell response. J Leukoc Biol, 73, 91-99.

CHUA, K. Y., STEWART, G. A., THOMAS, W. R., SIMPSON, R. J., DILWORTH, R. J., PLOZZA, T. M. & TURNER, K. J. (1988). Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. J.

Exp. Med., 167, 175-182.

CHUNSHENG, J., BRIGITTE, H., WOLFGANG, H., JOHANNES, S. & FRIEDRICH, A. (2008). Affinity of IgE and IgG against cross-reactive carbohydrate determinants on plant and insect glycoproteins. The Journal of allergy and clinical immunology, 121, 185-190.e2.

COMMINS, S. & PLATTS-MILLS, T. (2010). Allergenicity of Carbohydrates and Their Role in Anaphylactic Events. Current Allergy and Asthma Reports, 10, 29-33.

COMMINS, S. P. & PLATTS-MILLS, T. A. E. (2009). Anaphylaxis syndromes related to a new mammalian cross-reactive carbohydrate determinant. Journal of Allergy and Clinical Immunology, 124, 652-657.

CREGG, J. M. (2007). Introduction. Pichia Protocols.

CUSTOVIC, A., TAGGART, S. C. O., FRANCIS, H. C., CHAPMAN, M. D. & WOODCOCK, A. (1996). Exposure to house dust mite allergens and the clinical activity of asthma. Journal of Allergy and Clinical Immunology, 98, 64-72.

DAVID, D. C. (2010). Overview of the immune response. The Journal of allergy and clinical immunology, 125, S3-S23.

DE HALLEUX, S., STURA, E., VANDERELST, L., CARLIER, V., JACQUEMIN, M. & SAINT-REMY, J.-M. (2006). Three-dimensional structure and IgE-binding properties of mature fully active Der p 1, a clinically relevant major allergen. Journal of Allergy and Clinical Immunology, 117, 571-576.

DEB, R., SHAKIB, F., REID, K. & CLARK, H. (2007). Major house dust mite allergens Der p 1 and Der f 1 degrade and inactivate lung surfactant proteins -A and -D. J. Biol. Chem., 51, 36808-19.

DESLEE, G. T., CHARBONNIER, A.-S., HAMMAD, H., ANGYALOSI, G., TILLIE-LEBLOND, I., MANTOVANI, A., TONNEL, A. E.-B. & PESTEL, J. (2002) Involvement of the mannose receptor in the uptake of der p 1, a major mite allergen, by human dendritic cells. Journal of Allergy and Clinical Immunology, 110, 763-770.

DREBORG, S. (2003). Dietary prevention of allergy, atopy, and allergic diseases. Journal of Allergy and Clinical Immunology, 111, 467-470.

EBNER, C., SCHENK, S., NAJAFIAN, N., SIEMANN, U., STEINER, R., FISCHER, G. W., HOFFMANN, K., SZEPFALUSI, Z., SCHEINER, O. & KRAFT, D. (1995). Nonallergic individuals

recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. J Immunol, 154, 1932-1940.

ERBACHER, A., GIESEKE, F., HANDGRETINGER, R. & MÜLLER, I. (2009). Dendritic cells: Functional aspects of glycosylation and lectins. Human Immunology, 70, 308-312.

FÖTISCH, K. & VIETHS , S. (2001). N- and O-linked oligosaccharides of allergenic glycoproteins. Glycoconjugate Journal, 18, 373-390.

FRIEND SL, H. S., NELSON A, FOXWORTHE D, WILLIAMS DE,FARR A (1994). A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. Exp Hematol, 22, 321-328.

FURMONAVICIENE, R., GHAEMMAGHAMI, A. M., BOYD, S. E., JONES, N. S., BAILEY, K., WILLIS, A. C., SEWELL, H. F., MITCHELL, D. A. & SHAKIB, F. (2007). The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. Clinical & Experimental Allergy, 37, 231-242.

FURMONAVICIENE, R., SUTTON, B. J., GLASER, F., LAUGHTON, C. A., JONES, N., SEWELL, H. F. & SHAKIB, F. (2005). An attempt to define allergen-specific molecular surface features: a bioinformatic approach. Bioinformatics, 23, 4201-4204.

GAZI, U. & MARTINEZ-POMARES, L. (2009). Influence of the mannose receptor in host immune responses. Immunobiology, 214, 554-561.

GEIJTENBEEK, T. B. H., TORENSMA, R., VAN VLIET, S. J., VAN DUIJNHOVEN, G. C. F., ADEMA, G. J., VAN KOOYK, Y. & FIGDOR, C. G. (2000). Identification of DC-SIGN, a Novel Dendritic Cell-Specific ICAM-3 Receptor that Supports Primary Immune Responses. Cell, 100, 575-585.

GHAEMMAGHAMI, A. M., GOUGH, L., SEWELL, H. F. & SHAKIB, F. (2002). The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. Clinical & Experimental Allergy, 32, 1468-1475.

GHAEMMAGHAMI, A. M., ROBINS, A., GOUGH, L., SEWELL, H. F. & SHAKIB, F. (2001). Human T cell subset commitment

determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN- gamma. Eur. J. Immunol., 31, 1211–1216.

GOLL, D. E., THOMPSON, V. F., LI, H., WEI, W. E. I. & CONG, J. (2003). The Calpain System.

GOULD, H. J. & SUTTON, B. J. (2008). IgE in allergy and asthma today. Nat Rev Immunol, 8, 205-217.

GUY, B. M., EUAN, R. T., BRETT, G. T., SUSIE, W., JENNIFER, K. P. & ANN, J. W. (1995). Mite allergen (Der p 1) concentration in houses and its relation to the presence and severity of asthma in a population of Sydney schoolchildren. The Journal of allergy and clinical immunology, 96, 441-448.

HAMILTON, S. R. & GERNGROSS, T. U. (2007). Glycosylation engineering in yeast: the advent of fully humanized yeast. Current Opinion in Biotechnology, 18, 387-392.

HAMMAD, H. & LAMBRECHT, B. N. (2008). Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nat Rev Immunol, 8, 193-204.

HILMENYUK, T., BELLINGHAUSEN, I., HEYDENREICH, B., ILCHMANN, A., TODA, M., GRABBE, S. & SALOGA, J. (2010).

Effects of glycation of the model food allergen ovalbumin on antigen uptake and presentation by human dendritic cells. Immunology, 129, 437-445.

HSU SC, C. C., TSAI SH, KAWASAKI H, HUNG CH, CHU YT, CHANG HW, ZHOU Y, FU J, PLUNKETT B, SU SN, VIETHS S, LEE RT, LEE YC, HUANG SK. (2010). Functional interaction of common allergens and a C-type lectin receptor, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), on human dendritic cells. J Bio Chem, 285, 7903-10.

HUBY, R. D. J., DEARMAN, R. J. & KIMBER, I. (2000). Why Are Some Proteins Allergens? Toxicological Sciences. 55, 235-246.

ISHIHARA, H., TAKAHASHI, N., OGURI, S. & TEJIMA, S. (1979). Complete structure of the carbohydrate moiety of stem bromelain. An application of the almond glycopeptidase for structural studies of glycopeptides. Journal of Biological Chemistry, 254, 10715-10719.

JACQUET, A., MAGI, M., PETRY, H. & BOLLEN, A. (2002). High-level expression of recombinant house dust mite allergen Der p 1 in Pichia pastoris. Clinical & Experimental Allergy, 32, 1048-1053.

JOHANSSON, S. G. O., BIEBER, T., DAHL, R., FRIEDMANN, P. S., LANIER, B. Q., LOCKEY, R. F., MOTALA, C., ORTEGA MARTELL, J. A., PLATTS-MILLS, T. A. E., RING, J., THIEN, F., VAN CAUWENBERGE, P. & WILLIAMS, H. C. (2004). Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. Journal of Allergy and Clinical Immunology, 113, 832-836.

KALINSKI, P., HILKENS, C. M. U., WIERENGA, E. A. & KAPSENBERG, M. L. (1999). T-cell priming by type-1and type-2 polarized dendritic cells: the concept of a third signal. Immunology Today, 20, 561-567.

KAPSENBERG, M. L., HILKENS, C. M. U., VAN DER POUW KRAAN, T. C. M. T., WIERENGA, E. A. & KALINSKI, P. (2000). Atopic Allergy: A Failure of Antigen-Presenting Cells to Properly Polarize Helper T Cells? Am. J. Respir. Crit. Care Med., 162, 76S-80.

KAY, A. B. (2000). Overview of 'Allergy and allergic diseases: with a view to the future'. Br Med Bull, 56, 843-864.

KERRIGAN, A. M. & BROWN, G. D. (2009). C-type lectins and phagocytosis. Immunobiology, 214, 562-575.

KIM, H. Y., DEKRUYFF, R. H. & UMETSU, D. T. (2010). The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nat Immunol, 11, 577-584.

KIMURA Y, K. M., MAEDA M, OKANO M, YOKOYAMA M, KINO K. (2008). Glycoform analysis of Japanese cypress pollen allergen, Cha o 1: a comparison of the glycoforms of cedar and cypress pollen allergens. Biosci Biotechnol Biochem, 72, 485-491.

KOLARICH, D. & ALTMANN, F. (2000). N-Glycan Analysis by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Electrophoretically Separated Nonmammalian Proteins: Application to Peanut Allergen Ara h 1 and Olive Pollen Allergen Ole e 1. Analytical Biochemistry, 285, 64-75.

LAUER, I. A., FOETISCH, K.A , KOLARICH, D.B , BALLMER-WEBER, B.K.C , CONTI, A.D , ALTMANN, F.B , VIETHS, S.A , SCHEURER, S.A (2004). Hazelnut (Corylus avellana) vicilin Cor a 11: Molecular characterization of a glycoprotein and its allergenic activity. Biochemical Journal 383, 327-334.

LEONARD R, P. B., HIMLY M, KAAR W, WOPFNER N, KOLARICH D, VAN REE R, EBNER C, DUUS JØ, FERREIRA F, ALTMANN F. (2005). Two novel types of O-glycans on the

mugwort pollen allergen Art v 1 and their role in antibody binding. J Biol Chem, 280, 7932-40.

LI, Y.-L., LI, H.-J., JI, F., ZHANG, X., WANG, R., HAO, J.-Q., BI, W.-X. & DONG, L. (2010). Thymic Stromal Lymphopoietin Promotes Lung Inflammation Through Activation of Dendritic Cells. Journal of Asthma, 47, 117-123.

LIPNIUNAS, P., ANGEL, A.-S., ERLANSSON, K., LINDH, F. & NILSSON, B. (1992). Mass spectrometry of high-mannose oligosaccharides after trifluoroacetolysis and periodate oxidation. Analytical Biochemistry, 200, 58-67.

LIU H , P. H., CAI SX , CHEN ZW , ZHENG XF , YANG HT , XIAO ZY. (2005). The effect of fermentation conditions on glycosylation of recombinant human interferon omega in yeast Pichia pastoris. Journal of Biotechnology. 21, 107-12

MALIK, A., ARIF, S. A. M., AHMAD, S. & SUNDERASAN, E. (2008). A molecular and in silico characterization of Hev b 4, a glycosylated latex allergen. International Journal of Biological Macromolecules, 42, 185-190.

MARI, A. (2002). IgE to Cross-Reactive Carbohydrate Determinants: Analysis of the Distribution and Appraisal of

the in vivo and in vitro Reactivity. International Archives of Allergy and Immunology, 129, 286-295.

MENO, K., THORSTED, P. B., IPSEN, H., KRISTENSEN, O., LARSEN, J. N., SPANGFORT, M. D., GAJHEDE, M. & LUND, K. (2005). The Crystal Structure of Recombinant proDer p 1, a Major House Dust Mite Proteolytic Allergen. J Immunol, 175, 3835-3845.

MINTON, K. (2008). Allergy and Asthma: What 'drives' IL-4 versus IL-13 signalling? Nat Rev Immunol, 8, 166-167.

MITSUHIRO, O., KOHSUKE, K., TERUAKI, T., HISASHI, H., TERUHIRO, O., TADASHI, Y., MINEHIKO, Y. & KAZUNORI, N. (2001). Roles of carbohydrates on Cry j 1, the major allergen of Japanese cedar pollen, in specific T-cell responses. The Journal of allergy and clinical immunology, 108, 101-108.

MIYATA, M., NAKAMURA, Y., SHIMOKAWA, N., OHNUMA, Y., KATOH, R., MATSUOKA, S., OKUMURA, K., OGAWA, H., MASUYAMA, K. & NAKAO, A. (2009). Thymic stromal lymphopoietin is a critical mediator of IL-13-driven allergic inflammation. European Journal of Immunology, 39, 3078-3083.

NAVARRO, R. P., SCHAECHER, K. L. & RICE, G. K. (2007). Asthma management guidelines: updates, advances, and new options. J Manag Care Pharm, 13, S3-11.

NISHIYAMA, C., FUKADA, M., USUI, Y., IWAMOTO, N., YUUKI, T., OKUMURA, Y. & OKUDAIRA, H. (1995). Analysis of the IgE-epitope of Der f 2, a major mite allergen, by in vitro mutagenesis. Mol Immunol, 32, 1021-9.

OKANO, M., SATOSKAR, A. R., NISHIZAKI, K., ABE, M. & HARN, D. A., JR. (1999a). Induction of Th2 Responses and IgE Is Largely Due to Carbohydrates Functioning as Adjuvants on Schistosoma mansoni Egg Antigens. J Immunol, 163, 6712-6717.

OKANO, M., SATOSKAR, A., HARN, D., NISHIZAKI, K., OKANO, M., MASUDA, Y. & YOSHINO, T. (1999b) Involvement of carbohydrate on phospholipase A2, a bee venom allergen, in in vivo antigen specific IgE synthesis in mice. Allergy, 54, 811-818.

OLIVER SCHULZ, H. F. S., AND FAROUK SHAKIB. (1998). Proteolytic Cleavage of CD25, the α Subunit of the Human T Cell Interleukin 2 Receptor, by Der p 1, a Major Mite Allergen with Cysteine Protease Activity JEM, 187, 271-275.

O'NEIL, S. E., HEINRICH, T. K., HALES, B. J., HAZELL, L. A., HOLT, D. C., FISCHER, K. & THOMAS, W. R. (2006). The chitinase allergens Der p 15 and Der p 18 from Dermatophagoides pteronyssinus. Clinical & Experimental Allergy, 36, 831-839.

PASCHINGER, K., FABINI, G., SCHUSTER, D., RENDIĆ D & WILSON, I. B. (2005). Definition of immunogenic carbohydrate epitopes. Acta Biochim Pol, 52, 629-32.

PERLIN, A. S. (2006). Glycol-Cleavage Oxidation. In: DEREK, H. (ed.) Advances in Carbohydrate Chemistry and Biochemistry. Academic Press.

PETERSEN, SCHRAMM, SCHLAAK & BECKER (1998). Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen. Clinical & Experimental Allergy, 28, 315-321.

PHILIP E. THORPE, P. M. W., PHILLIP P. KNOWLES, MICHELE G. RELF, ALEX N. F. BROWN, GRAHAM J. WATSON, DAVID C. BLAKEY, AND DAVID R. NEWELL (1988). Improved Antitumor Effects of Immunotoxins Prepared with Deglycosylated Ricin A-Chain and Hindered Disulfide Linkages Cancer Res 48, 6396-6403.

PLASENCIA, M. D., ISAILOVIC, D., MERENBLOOM, S. I., MECHREF, Y. & CLEMMER, D. E. (2008). Resolving and Assigning N-Linked Glycan Structural Isomers from Ovalbumin by IMS-MS. Journal of the American Society for Mass Spectrometry, 19, 1706-1715.

PLATTS-MILLS, T., LEUNG, D. Y. & SCHATZ, M. (2007). The role of allergens in asthma. Am Fam Physician, 76, 675-80.

POLTL, G., AHRAZEM, O., PASCHINGER, K., IBANEZ, M. D., SALCEDO, G. & WILSON, I. B. H. (2007). Molecular and immunological characterization of the glycosylated orange allergen Cit s 1. Glycobiology, 17 (2): 220-230.

RASHEEDI, S., HAQ, S., KHAN, R., (2003). Guanidine hydrochloride denaturation of glycosylated and deglycosylated stem bromelain. Biochemistry, 68 (10): 1097-1100.

RAY, R. J., FURLONGER, C., WILLIAMS, D. E. & PAIGE, C. J. (1996). Characterization of thymic stromal-derived lymphopoietin (TSLP) in murine B cell development in vitro. European Journal of Immunology, 26, 10-16.

REINER., S. (2007). Development in motion: T helper cells at work. Cell, 129, 33-6.

RICHARDSON, G., EICK, S. & JONES, R. (2005). How is the indoor environment related to asthma? literature review. Journal of Advanced Nursing, 52, 328-339.

ROBINSON, C., KALSHEKER, N. A., SRINIVASAN, N., KING, C. M., GARROD, D. R., THOMPSON, P. J. & STEWART, G. A. (1997). On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. Clinical & Experimental Allergy, 27, 10-21.

ROGERS, P. R. & CROFT, M. (1999). Peptide Dose, Affinity, and Time of Differentiation Can Contribute to the Th1/Th2 Cytokine Balance. J Immunol, 163, 1205-1213.

ROGGEN, E. L., LINDSTEDT, M., BORREBAECK, C. & VERHEYEN, G. R. (2006). Interactions between dendritic cells and epithelial cells in allergic disease. Toxicology Letters, 162, 71-82.

ROITT, I. M. & DELVES, P. J. (2001). Roitt's Essential Immunology, Blackwell Science.

ROYER, P.-J., EMARA, M., YANG, C., AL-GHOULEH, A., TIGHE, P., JONES, N., SEWELL, H. F., SHAKIB, F., MARTINEZ-POMARES, L. & GHAEMMAGHAMI, A. M. (2010). The Mannose

Receptor Mediates the Uptake of Diverse Native Allergens by Dendritic Cells and Determines Allergen-Induced T Cell Polarization through Modulation of IDO Activity. J Immunol, 185: 1522-31.

RUSSANO, A. M., AGEA, E., CASCIARI, C., BENEDICTIS, F. M. D. & SPINOZZI, F. (2008). Complementary roles for lipid and protein allergens in triggering innate and adaptive immune systems. Allergy, 63(11):1428-37

SAITO, H. (2007). Allergy and hypersensitivity -- airway inflammation and remodeling. Current Opinion in Immunology, 19: 674-5.

SANDRINE, J., DENISE-ANNE, M.-V. & BERNARD, E. B. (2009). Mammalian meat–induced anaphylaxis: Clinical relevance of anti–galactose-α-1,3-galactose IgE confirmed by means of skin tests to cetuximab. The Journal of allergy and clinical immunology, 124, 603-605.

SEPPÄLÄ, U., SELBY, D., MONSALVE, R., KING, T. P., EBNER, C., ROEPSTORFF, P. & BOHLE, B. (2009). Structural and immunological characterization of the N-glycans from the major yellow jacket allergen Ves v 2: The N-glycan structures

are needed for the human antibody recognition. Molecular Immunology, 46, 2014-2021.

SHAKIB, F., GHAEMMAGHAMI, A. M. & SEWELL, H. F. (2008). The molecular basis of allergenicity. Trends in Immunology, 29, 633-642.

SHAKIB, F., SCHULZ, O. & SEWELL, H. (1998). A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. Immunology Today, 19, 313-316.

SHARMA, S., LACKIE, P. M. & HOLGATE, S. T. (2003). Uneasy breather: the implications of dust mite allergens. Clinical & Experimental Allergy, 33, 163-165.

SHREFFLER, W. G., CASTRO, R. R., KUCUK, Z. Y., CHARLOP-POWERS, Z., GRISHINA, G., YOO, S., BURKS, A. W. & SAMPSON, H. A. (2006). The Major Glycoprotein Allergen from Arachis hypogaea, Ara h 1, Is a Ligand of Dendritic Cell-Specific ICAM-Grabbing Nonintegrin and Acts as a Th2 Adjuvant In Vitro. J Immunol, 177, 3677-3685.

SHREFFLER, W. G., CASTRO, R. R., KUCUK, Z. Y., CHARLOP-POWERS, Z., GRISHINA, G., YOO, S., BURKS, A. W. & SAMPSON, H. A. (2006). The Major Glycoprotein Allergen from Arachis hypogaea, Ara h 1, is a Ligand of Dendritic Cell-

Specific ICAM-Grabbing Nonintegrin and Acts as a Th2 Adjuvant In Vitro. J Immunol, 177, 3677-3685.

SINGH, S. K., STEPHANI, J., SCHAEFER, M., KALAY, H., GARCÍA-VALLEJO, J. J., DEN HAAN, J., SAELAND, E., SPARWASSER, T. & VAN KOOYK, Y. (2009). Targeting glycan modified OVA to murine DC-SIGN transgenic dendritic cells enhances MHC class I and II presentation. Molecular Immunology, 47, 164-174.

SINHA, J., PLANTZ, B. A., INAN, M. & MEAGHER, M. M. (2005). Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast Pichia pastoris: Case study with recombinant ovine interferon-τ. Biotechnology and Bioengineering, 89, 102-112.

SL., R. (2007). Development in motion: T helper cells at work. Cell, 129, 33-6.

SMAGUR, J., GUZIK, K., BZOWSKA, M., KUZAK, M., ZAREBSKI, M., KANTYKA, T., WALSKI, M., GAJKOWSKA, B. & POTEMPA, J. (2009). Staphylococcal cysteine protease staphopain B (SspB) induces rapid engulfment of human neutrophils and monocytes by macrophages. Biological Chemistry, 390, 361-371.

SOUMELIS, V., RECHE, P. A., KANZLER, H., YUAN, W., EDWARD, G., HOMEY, B., GILLIET, M., HO, S., ANTONENKO, S., LAUERMA, A., SMITH, K., GORMAN, D., ZURAWSKI, S., ABRAMS, J., MENON, S., MCCLANAHAN, T., WAAL-MALEFYT, R. D., BAZAN, F., KASTELEIN, R. A. & LIU, Y.-J. (2002). Human epithelial cells trigger dendritic cell-mediated allergic inflammation by producing TSLP. Nat Immunol, 3, 673-680.

SPIEKSMA FT, S.-B. M. (1967) "The mite fauna of house dust with particular reference to the house-dust mite Dermatophagoides pteronyssinus ". Acarologia, 1, 226-41.

STAUDACHER, E., ALTMANN, F., WILSON, I. B. H. & MÄRZ, L. (1999). Fucose in N-glycans: from plant to man. Biochimica et Biophysica Acta (BBA) - General Subjects, 1473, 216-236.

TADA T, T. T., OKUMURA K, NONAKA M, TOKUHISA T (1978). Two distinct types of helper T cells involved in the secondary antibody response: independent and synergistic effects of Ia- and Ia+ helper T cells. J Exp Med, 147, 446-58.

TAKAI, T., KATO, T., YASUEDA, H., OKUMURA, K. & OGAWA, H. (2005). Analysis of the structure and allergenicity of recombinant pro- and mature Der p 1 and Der f 1: Major

conformational IgE epitopes blocked by prodomains. Journal of Allergy and Clinical Immunology, 115, 555-563.

TANAKA, J., WATANABE, N., KIDO, M., SAGA, K., AKAMATSU, T., NISHIO, A. & CHIBA, T. (2009). Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions. Clinical & Experimental Allergy, 39, 89-100.

TAYLOR, P. R., GORDON, S. & MARTINEZ-POMARES, L. (2005). The mannose receptor: linking homeostasis and immunity through sugar recognition. Trends in Immunology, 26, 104-110.

THOMAS W. R. , SMITH W. , HALES B. J. , MILLS, K. L. & M., O. B. R. (2002). Characterization and Immunobiology of House Dust Mite Allergens. Int Arch Allergy Immunol, 129, 1-18.

THOMAS, W. R. & SMITH, W. (1998). House-dust-mite allergens. Allergy, 53, 821-832.

TSAI, L. C., PENG, H. J., LEE, C. S., CHAO, P. L., TANG, R. B., TSAI, J. J., SHEN, H. D., HUNG, M. W. & HAN, S. H. (2005). Molecular cloning and characterization of full-length cDNAs

encoding a novel high-molecular-weight Dermatophagoides pteronyssinus mite allergen, Der p 11. Allergy, 60, 927-937.

TURVEY, S. E. & H., B. D. (2010). Innate immunity. The Journal of allergy and clinical immunology, 125, S24-S32.

VALENTA, R. & KRAFT, D. (1995) Recombinant allergens for diagnosis and therapy of allergic diseases. Current Opinion in Immunology, 7, 751-756.

VAN OORT, E., DE HEER, P. G., VAN LEEUWEN, W. A., DERKSEN, N. I. L., MULLER, M., HUVENEERS, S., AALBERSE, R. C. & VAN REE, R. (2002). Maturation of Pichia pastoris-derived recombinant pro-Der p 1 induced by deglycosylation and by the natural cysteine protease Der p 1 from house dust mite. European Journal of Biochemistry, 269, 671-679.

VAN REE, R., CABANES-MACHETEAU, M., AKKERDAAS, J., MILAZZO, J.-P., LOUTELIER-BOURHIS, C., RAYON, C., VILLALBA, M., KOPPELMAN, S., AALBERSE, R., RODRIGUEZ, R., FAYE, L. Ì. C. & LEROUGE, P. (2000). Beta (1,2)-Xylose and alpha (1,3)-Fucose Residues Have a Strong Contribution in IgE Binding to Plant Glycoallergens. J Biol Chem. 275, 11451-11458.

VERVECKEN, W., CALLEWAERT, N., KAIGORODOV, V., GEYSENS, S. & CONTRERAS, R. (2007). Modification of the N-Glycosylation Pathway to Produce Homogeneous, Human-Like Glycans Using GlycoSwitch Plasmids. Pichia Protocols. 389, 119-138.

VINEY, J. L. (1999) Dendritic cell subsets: the ultimate T cell differentiation decision makers? *Gut,* 45**,** 640-641.

VOORHORST R, S. F. (1969). Recent progress in the house dust mite problem. Acta Allergol., 24, 115-23.

WAN, WINTON, SOELLER, GRUENERT, THOMPSON, CANNELL, STEWART, GARROD & ROBINSON (2000). Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. Clinical & Experimental Allergy, 30, 685-698.

WANG, J. & XING, F. (2008). Human TSLP-Educated DCs. Cell Mol Immunol, 5, 99-106.

WANG, J., ZHANG, Y., WEI, J., ZHANG, X., ZHANG, B., ZHU, Z., ZOU, W., WANG, Y., MOU, Z., NI, B. & WU, Y. (2007). Lewis X oligosaccharides targeting to DC-SIGN enhanced

antigen-specific immune response. Immunology, 121, 174-182.

WANG, Y. H. & LIU, Y. J. (2009). Thymic stromal lymphopoietin, OX40-ligand, and interleukin-25 in allergic responses. Clinical & Experimental Allergy, 39, 798-806.

WEGHOFER, M., THOMAS, W. R., PITTNER, G., HORAK, F., VALENTA, R. & VRTALA, S. (2005). Comparison of purified Dermatophagoides pteronyssinus allergens and extract by two-dimensional immunoblotting and quantitative immunoglobulin E inhibitions. Clinical & Experimental Allergy, 35, 1384-1391.

WIERENGA, E. A., SNOEK, M., DE GROOT, C., CHRETIEN, I., BOS, J. D., JANSEN, H. M. & KAPSENBERG, M. L. (1990). Evidence for compartmentalization of functional subsets of CD4+ T lymphocytes in atopic patients. J Immunol, 144, 4651-4656.

WILLS-KARP, M., NATHAN, A., PAGE, K. & KARP, C. L. (2009). New insights into innate immune mechanisms underlying allergenicity. Mucosal Immunol, 3, 104-110.

YANG, L., KAJIURA, H., SUZUKI, K., HIROSE, S., FUJIYAMA, K. & TAKAIWA, F. (2008). Generation of a transgenic rice

seed-based edible vaccine against house dust mite allergy. Biochemical and Biophysical Research Communications, 365, 334-339.

YOKOYAMA, S. (2003). Protein expression systems for structural genomics and proteomics. Current Opinion in Chemical Biology, 7, 39-43.

ZHANG, Y., LIU, R. & WU, X. (2007). The proteolytic systems and heterologous proteins degradation in the methylotrophic yeast Pichia pastoris. Annals of Microbiology, 57, 553-560.

ZIEGLER, S. F. & ARTIS, D. (2010). Sensing the outside world: TSLP regulates barrier immunity. Nat Immunol, 11, 289-293.